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THE UNIVERSITY OF ALBERTA

ROLE OF FATTY ACID AND GLUCOSE METABOLISM IN DEPRESSED CARDIAC FUNCTION IN DIABETIC RATS

BY

STEPHEN RICHARD WALL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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EDMONTON, ALBERTA

FALL, 1988

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ABSTRACT

The acute and chronic effects of elevated fatty acid levels on myocardial glucose oxidation and cardiac function in diabetes were investigated. Administration of agents which activate pyruvate dehydrogenase (PDH) by inhibiting fatty acid oxidation at carnitine palmitoyltransferase I (CPT I) can reverse depressed myosin ATPase activity in diabetic rat hearts via regulation of myosin isoenzyme production. In the first study, chronically diabetic rats were administered dichloroacetate (DCA), an agent which directly stimulates PDH, for 5 weeks, after which glucose oxidation rates were measured as $^{14}CO_2$ production from ^{14}C-glucose in fatty acid-perfused isolated working hearts. Depressed glucose oxidation rates in diabetic rat hearts were increased by DCA treatment. Ca^{2+}-activated myofibril ATPase activity, measured in hearts from the same animals, was depressed in untreated diabetics, but was completely normalized by DCA treatment. However, depressed cardiac function in diabetic rats was not increased by DCA treatment. In the absence of increase serum thyroxine in DCA-treated diabetics, these data suggest that a metabolic signal related to glucose oxidation regulates ATPase activity or cardiac myofibrils, probably by affecting myosin isozyme expression.

In the second study, the acute effects of fatty acids on glucose oxidation and function were studied in isolated working hearts from chronically diabetic rats. In control hearts, the presence of 1.2 mM palmitate in the perfusate produced a marked 13-fold decrease in glucose oxidation, to rates seen in diabetic rat

hearts perfused with glucose alone. The presence of palmitate resulted in almost complete suppression of glucose oxidation in diabetic rat hearts. Direct measurement of exogenous palmitate oxidation showed no difference between control and diabetic rat hearts. Cardiac function was reduced in diabetic rat hearts under both substrate conditions. Addition of the CPT I inhibitor, Etomoxir (10^{-6} M), significantly increased glucose oxidation rates in all conditions, but the increase was smaller in diabetic-palmitate perfused rat hearts. Etomoxir did not reduce exogenous palmitate oxidation rates or citrate levels. An increase in cardiac function, mainly due to increased heart rate, was observed after addition of Etomoxir to palmitate-perfused control and diabetic rat hearts. These data suggest that acutely reversible effects of fatty acids can contribute to reduced myocardial glucose oxidation and function in diabetes.

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CHAPTER 1: INTRODUCTION

Epidemiological studies demonstrate that the increased incidence of heart disease and incidence of congestive heart failure in patients with diabetes mellitus is partly attributable to an inherent cardiomyopathy independent of atherosclerosis (for reviews, see Kannel and McGee, 1979; Kannel, 1985; Kereiakes et al., 1984). For instance, survival after myocardial infarction is reduced in patients with diabetes (Kannel and McGee, 1979). Experimentally, a greater susceptibility to ischemic insult has been suggested by studies in isolated working hearts from diabetic rats (Feuvray et al., 1979; Hekimian and Feuvray, 1986). This may be related to fatty acids (Lopaschuk and Spafford, 1988a,b), which are increased in the blood and myocardium of the diabetic (Denton and Randle, 1967; Paulson and Crass, 1982; Murthy and Shipp, 1977; Lopaschuk and Tsang, 1987). Even in the absence of diabetes, perfusion of working hearts with fatty acids can increase the severity of ischemia (Liedtke et al., 1984; DeLeiris and Opie, 1978; Lopaschuk et al, 1988). In non-ischemic hearts, impaired cardiac performance in diabetics in response to high work demand has been demonstrated in both clinical studies, such as during exercise (Fisher et al., 1986, 1987; Vered et al., 1984; Mildenberger et al., 1984) and in isolated working rat heart preparations, where work was increased by increasing preload and/or afterload, or by cardiac pacing (Miller, 1979; Penparkgul et

al., 1980; Vadlamudi et al., 1982; Garber and Neely, 1983).

One experimental model used to demonstrate impaired cardiac performance in diabetics is the isolated working diabetic rat heart. At physiologic workloads, reductions in heart rate (HR) and peak systolic pressure (PSP) occur (Miller, 1979; Ingebretsen et al., 1980; Penparkgul et al., 1980; Vadlamudi et al. 1982; Garber and Neely, 1983). Contractile function (as measured by changes in left ventricular dP/dt) is also impaired in response to an increasing range of left atrial filling pressure (Miller, 1979; Penparkgul et al., 1980: Vadlamudi et al., 1982; Ingebretsen et al., 1980; Barbee et al., 1988). At maximal workloads elicited by increasing left atrial filling pressure and aortic afterload, HRXPSP is reduced as a result of lower HR and PSP. Depressed left ventricular +dP/dt at high workload cannot be corrected in diabetic rat hearts even when HR and PSP are normalized by electrical pacing and adjustment of aortic afterload (Garber and Neely, 1983). Insulin injections can prevent the functional changes, or reverse such changes occurring after about 6 weeks of diabetes, if insulin treatment is continued for 4 weeks (Tahiliani et al., 1983; Fein et al., 1981; 1984; Garber et al., 1983; Rubinstein et al., 1984). Functional changes occurring over 5 months of diabetes could not be reversed by 4 weeks of insulin treatment, however (Tahiliani and McNeill, 1984). Reduced contractility has also been demonstrated directly in isolated papillary muscle preparations as prolonged time to peak tension and time to half relaxation in isometric studies, and prolonged time to peak shortening and decreased shortening velocity during isotonic contraction (Fein et al., 1980).

Biochemical Mechanisms of Reduced Cardiac Function in Diabetes:

Several biochemical mechanisms can be invoked to account for the functional changes that occur in the diabetic rat myocardium. The decreased heart rate appears as early as 48 hours after induction of chemical diabetes and can be noted in the spontaneously diabetic "BB" Wistar rat as soon as 24 hours after insulin withdrawal (Garber and Neely, 1983; Lopaschuk and Spafford, 1988a; Lopaschuk and Tsang, 1987). The reduction in heart rate may be related to altered electrophysiology of the systems responsible for generation of the pacemaker potential. The membrane properties which determine the pacemaker behaviour are changes in permeability to K^+, Na^+, and/or Ca^{2+} during diastole. In this regard, Sauviat and Feuvray (1986) have demonstrated prolonged action potential duration and repolarization time, as well as greater reductions in maximal rate of action potential development in response to decreasing external Ca^{2+} concentration, in diabetic rat papillary muscles. Other authors have also noted greater sensitivity to external Ca^{2+} deprivation (Bielefeld et al., 1983) or altered function of Na^+-K^+ATPase (Fein et al., 1983; Ku and Sellers, 1982; Pierce and Dhalla, 1983), which may also be related to decreased intrinsic heart rate in diabetic rats. Depressed sarcolemma or sarcoplasmic reticulum Ca^{2+} fluxes or reuptake or binding by these systems (Lopaschuk et al., 1983; Pierce et al., 1983), may contribute to impaired activation or relaxation of cardiac muscle in the diabetic.

A major determinant of contractility of cardiac muscle is the myosin ATPase activity. This is demonstrated by good correlations between maximal shortening velocity of isolated papillary muscles

(Vmax) and activity of myosin ATPase (Hamrell and Low, 1978; Maugham et al., 1979). Cardiac myosin in rats exists as three isoenzymes with varying ATPase activity (ATPase activity increases from V3-V1) which are distinguishable by native pyrophosphate gel electrophoresis (Hoh et al., 1978). Two myosin heavy chain (MHC) types (α and β) which are encoded by distinct genes (Mahdavi et al., 1984) form three possible MHC dimers in myosin. Thus, V1 myosin contains an $\alpha\alpha$ homodimer, V2 an$\alpha\beta$ heterodimer, and V3 a $\beta\beta$ homodimer (Hoh et al., 1979). The myosin isozyme distribution can be correlated with mechanical indices of contractility (Schwartz et al., 1981; Ebrecht et al., 1982; Pagani and Julian, 1984; Lecarpentier et al., 1987; Holubarsch et al., 1985). Measurement of ATPase activity of intact myofibrils is suggested to be a physiologically relevant biochemical indicator of myocardial contractility, since the coordinated regulation of myosin ATPase by Ca^{2+} and the thin filament are accounted for (Scheuer and Bhan, 1979). Cardiac myofibril ATPase activity can be correlated with myosin isozyme distribution and contractility of isolated trabecular preparations, under normal conditions and in various experimental situations in which myosin isozyme switches occur (Rupp, 1981, 1982; Holubarsch et al., 1985). As mentioned, reduced contractility in diabetes has been demonstrated in the isolated working heart and in isolated papillary muscle preparations. Thus, it is not surprising that diabetic rat hearts have depressed ATPase activity of actomyosin, myosin, and myofibrils (Malhotra et al., 1981; Dillmann, 1980; Pierce and Dhalla, 1981; Garber and Neely, 1983; MacLean et al., 1987). This appears to be largely due to a shift in cardiac myosin isoform predominance from

V1 to V3 (Dillman, 1980), although the role of other components of the myofibril has not been examined.

There is controversy regarding the status of myocardial high energy phosphates levels in diabetes. Allison (1976) demonstrated a reduction in in vivo ATP levels in diabetic rats which he attributed to decreased red blood cell 2,3-diphosphoglycerate levels, which would increase oxygen affinity for hemoglobin, and thereby reduce oxygen delivery. In this and other studies of acutely diabetic rats, ATP levels could be normalized by provision of high glucose concentrations or insulin in vitro (Allison, 1976; Miller, 1979; Garber and Neely, 1983). However, Opie et al. (1979) observed decreased ATP and creatine phosphate (CP) levels in hearts from rats with diabetes of 3 days or more duration, even when hearts were perfused in the presence of insulin. Rosen et al. (1986) observed decreased myocardial levels of ATP and CP in vivo and reduced CP levels in hearts perfused in the Langendorff mode with glucose and octanoate in chronically diabetic versus control rats. They suggested that there was a loss of adenine nucleotides and an impairment of the creatine kinase reaction. Dillmann et al. (APS abstr.1987 San Diego) also observed reduced creatine kinase at both protein and mRNA levels. As described in the next section, levels of fatty acid intermediates, long chain acylcarnitine and long chain acyl CoA can be increased in diabetic rat heart (Feuvray et al. 1979). Pieper et al. (1984a,b) and Paulson and Shug (1982) suggest that ATP levels are reduced in perfused diabetic rat heart due to elevated long chain acyl CoA levels, which are suggested to inhibit the mitochondrial adenine nucleotide translocase (Paulson and Shug,

1984), thereby reducing ATP export from the mitochondrion. Pieper et al. (1984a) observed that in Langendorff-perfused hearts from acutely diabetic rats, ATP levels were reduced as the concentration of palmitate in the perfusate was increased, and that ATP levels were inversely correlated with tissue long chain acyl CoA levels. However, tissue ATP levels were lov even in control hearts. They claimed that administration of carnitine to normalize decreased myocardial carnitine levels in the diabetic would reduce long chain acyl CoA by facilitating transfer of the acyl group to carnitine. The resultant drop in long chain acyl CoA levels would relieve inhibition of the adenine nucleotide translocase, thus alleviating diminished ATP levels. The mechanism by which carnitine improves metabolism and performance in hearts from diabetic rats (Paulson et al., 1984), or cardiomyopathic Syrian hamsters (Whitmer, 1987), or during acute myocardial ischemia (Paulson et al., 1986) may be related to its ability to decrease the mitochondrial ratio of acetyl CoA/CoA. Carnitine, by facilitating acetylcarnitine efflux from the mitochondrion (Lysiak et al., 1988), may increase glucose oxidation by subsequently reactivating the PDH complex (Randle et al., 1984). The concept of long chain acyl CoA in inhibiting the adenine nucleotide translocase has been examined mainly with reference to the decrease in ATP levels occurring in myocardial ischemia. The original concept was that the decline in ATP during ischemia was exascerbated by inhibition of the translocase as long chain acyl CoA accumulated. This has received considerable criticism, however. Some objections are that cytosolic levels of long chain acyl CoA may not be high enough to cause inhibition (LaNoue et al., 1981),

inhibition is not necessarily seen in non-ischemic tissue with high levels of long chain acyl CoA (Lochner et al., 1981), and inhibition by matrix-localized long chain acyl CoA is not always observed (LaNoue et al., 1981; Ho and Pande, 1974; Woldegiorgis et al., 1982; Paulson and Shug, 1984). Pande et al. (1984) suggest that a decrease in ATP levels must occur before binding of long chain acyl CoA to the translocase, and that binding serves to prevent further metabolism of fatty acids and accumulation of fatty acid intermediates. It remains to be resolved exactly what changes in myocardial high energy phosphate metabolism occur, and what effect of fatty acids may have in diabetes.

Myocardial Fatty Acid Metabolism in Diabetes:

In diabetes, the heart must depend more on fatty acids for provision of energy (Denton and Randle, 1967). This may have acute and chronic effects on cardiac function. Serum fatty acids and myocardial triacylglycerol concentrations are elevated in the diabetic (Denton and Randle, 1967; Murthy and Shipp, 1977; Paulson and Crass, 1982; Lopaschuk and Tsang, 1987). Myocardial levels of fatty acid intermediates, long chain acyl CoA and long chain acylcarnitine, are increased in the diabetic (Feuvray et al., 1979). These intermediates have been suggested to impair membrane function via their detergent-like effect on membranes (Katz and Messineo, 1981), and/or by inhibiting specific membrane-associated enzyme systems (Adams et al., 1979; Lopaschuk et al., 1983; Kakar et al., 1987; Paulson and Shug, 1984). High fatty acid levels may be acutely detrimental to function in the chronically diabetic rat heart, perhaps by inhibiting critical glucose metabolism required for high

work output. Lopaschuk and Spafford (1988b), in examining the effect of perfusion with fatty acids on failure rate in diabetic rat hearts during low-flow ischemia and cardiac pacing, observed that chronically diabetic rat hearts could not be paced at the same rate as controls if 1.2 mM palmitate was added to the perfusate.

As mentioned, myocardial triacylglycerol stores are increased in diabetes. While this has been attributed to elevated serum levels of fatty acids which can both increase triacylglycerol synthesis (Denton and Randle, 1967) and inhibit hydrolysis (Paulson and Crass, 1982; Murthy et al., 1983), it has been recently suggested that elevated levels of total CoA seen in diabetes increase triacylglycerol synthesis. Increased cytosolic levels of long chain acyl CoA, resulting from elevated circulating fatty acids and cytosolic CoA favours storage of fatty acids in complex lipids, as opposed to transport to the mitochondrion for oxidation (Lopaschuk et al., 1986, Lopaschuk and Tsang, 1987). Fatty acids which are stored esterified in triacylglycerols can be used for energy provision via mitochondrial oxidation. Work by Denton and Randle (1967) in which endogenous fatty acid oxidation rate was calculated from measured glucose utilization and total oxygen consumption demonstrates that fatty acid oxidation from stored triacylglycerols provides a greater proportion of energy in glucose-perfused diabetic than in control rat hearts. Paulson and Crass (1982), who measured $^{14}CO_2$ production from prelabelled triacylglycerol stores, also demonstrate that rates of fatty acid oxidation from stored triacylglycerols can vary depending on the concentration of exogenous fatty acids (Paulson and Crass, 1982) Rates of exogenous fatty acid oxidation can also vary

depending on the exogenous fatty acid concentration (Neely and Morgan, 1974). Therefore, levels of exogenous fatty acids and stored triacylglycerols are probably important determinants of myocardial fatty acid oxidation in the diabetic. Earlier studies suggested that rates of fatty acid oxidation from perfusate ^{14}C-labelled fatty acid were actually reduced in isolated hearts from alloxan diabetic rats (Kreisberg, 1966). However, when reduced work performed in diabetic hearts is accounted for, there is no difference in palmitate oxidation rates between control and spontaneously diabetic "BB" Wistar rat hearts (Lopaschuk and Tsang, 1987). This question had not yet been investigated in chemically diabetic (i.e., streptozotocin) diabetic rats.

Perhaps the most profound effect of an increased reliance of diabetic rat hearts on fatty acid metabolism is a reduction in myocardial glucose oxidation. Insulin deficiency results in decreased glucose uptake. In addition, elevated flux of fatty acids from serum and stored triacylglycerols reduces glucose utilization as a result of accumulation of intermediates of fatty acid oxidation. According to the classic concept of the glucose-fatty acid cycle (Randle et al., 1963), increased rates of fatty acid oxidation cause "unspanning" of the TCA cycle (Randle et al., 1970; Neely and Morgan, 1974). Increased citrate levels inhibit glycolysis at phosphofructokinase 1 (PFK 1) while increased acetyl CoA and ratio of acetyl CoA/CoA inhibits pyruvate oxidation at pyruvate dehydrogenase (PDH) (Neely et al., 1970; Randle, 1985, 1986; Randle et al., 1978; Randle et al., 1984). Addition of fatty acids to isolated perfused hearts from normal rats can suppress glucose oxidation at steps of

glucose uptake, glyco(geno)lysis, and pyruvate oxidation to rates seen in glucose-perfused diabetic rat hearts (Garland et al., 1962, 1964a,b; Newsholme and Randle, 1964; Randle et al., 1963, 1964). This suggests that reduced glucose oxidation in diabetic rat hearts may occur partly due to the acute effects of increased fatty acid oxidation. In addition to this, however, a more stable effect of diabetes reduces glucose oxidation at the PDH complex, and this may be mediated by chronically increased lipid metabolism. The percentage of the PDH complex in the active, dephosphorylated form (%PDHa) is regulated by reversible phosphorylation at three sites. This phosphorylation is under the influence of PDH kinase and PDH phosphatase. PDH kinase is stimulated by increasing the mitochondrial concentration ratios of acetyl CoA/CoA, NADH/NAD^+, and ATP/ADP, and inhibited by pyruvate. PDH phosphatase is stimulated by Ca^{2+} within a physiologic range and its effectiveness is reduced with increasing number of sites phosphorylated on PDH. The PDHa is also regulated by feedback inhibition from increasing product/substrate concentration ratios (see Randle et al., 1984; Kerbey et al., 1985 for reviews). The effect of oxidation of lipid fuels (fatty acids or ketone bodies) is to increase PDH kinase activity by increasing mitochondrial concentraiton ratios of acetyl CoA/CoA and possibly NADH/NAD^+. Hence, inhibition of carnitine palmitoyltransferase I (CPT I), the enzyme catalyzing the first step in the transport of activated long chain fatty acids into the mitochondrion for oxidation, can activate the PDH complex in perfused rat hearts. However, CPT I inhibition does not increase %PDHa to the same extent in diabetic rat heart as it does in controls perfused

with glucose and insulin (Caterson et al., 1982). The PDH complex also is resistant to activators such as dichloroacetate (DCA) and Ca^{2+} in isolated mitochondria from diabetic rat hearts (Kerbey et al., 1976, 1977; Hutson et al., 1978). The %PDHa appears to be reduced in diabetes due to increased PDH kinase activity, both intrinsic to the complex and in the form of a free "kinase/activator" protein. Thus, perfusion with fatty acids reduces the %PDHa to 7, whereas in diabetes the %PDHa is only 1-2. This increased phosphorylation of the PDH complex results in resistance to reactivation by PDH phosphatase (Randle et al., 1984; Kerbey et al., 1984). The net effect is that %PDHa is relatively lower in isolated perfused diabetic rat hearts than in normals when hearts are perfused under various conditions which change PDH activity, such as ischemia, high work, and perfusion with fatty acids or insulin (Randle et al., 1984). Perfusion with CPT I inhibitors such as tolbutamide and the phenylalkyloxirane carboxylic, POCA, can increase glucose oxidation in glucose-perfused diabetic rat heart to some extent (Tan et al., 1984; Rosen and Reinauer, 1984). However, the effectiveness of perfusion in the presence of CPT I inhibitors in increasing glucose oxidation in the diabetic rat heart has not been assessed in the presence of physiologic levels of fatty acids, which may override the inhibition of CPT I (Caterson et al., 1982). The effects of chronic administration of CPT I inhibitors on glucose oxidation, or %PDHa, in diabetic rat heart has similarly not been examined. Incubation of cultured hepatocytes for 21 hours with octanoate and glucagon at concentrations approximating in vivo levels during starvation or diabetes can increase PDH kinase activity (Fatania et al., 1986).

The long-term hormonal and/or metabolic factors in diabetes which regulate increased PDH kinase activity, and stable reduction of %PDHa in relation to decreased glucose oxidation in the myocardium, are incompletely understood.

The role of citrate in diabetes-induced inhibition of glycolysis should be reevaluated in relation to the importance of reductions in levels of fructose-2,6-bisphosphate (Fru-2,6-P2)(Sochor et al., 1984). Fru-2,6-P2, produced by the PFK 2 reaction, is the most potent known stimulator of PFK 1 (see Hue and Rider, 1987 for review). It acts by relieving inhibition due to citrate and ATP and has been suggested to be critical in maintaining physiologic rates of glycolysis in the working heart (Lawson and Uyeda, 1987; Narabayashi et al., 1985). However, the role of this compound in regulating PFK 1 in the presence of fatty acids or in diabetes has not been investigated.

Chronic administration of the CPT I inhibitor methylpalmoxirate can partially normalize the shift in myosin isozyme predominance and depressed myosin ATPase activity, and completely normalize depressed sarcoplasmic reticulum Ca^{2+} uptake seen in diabetic rat heart (Dillmann, 1985; Tahiliani and McNeill, 1985). It was not confirmed, however,if improvements in metabolism were accomplished by methylpalmoxirate in these studies. In any case, these biochemical changes in sarcoplasmic reticulum and myosin may not directly correlate with functional changes, as increases were not seen in variables such as papillary muscle shortening velocity (Popovich et al., 1985) or left ventricular developed pressure in isolated working hearts subjected to increasing left atrial filling

pressure (Tahiliani and McNeill, 1985).

Role of Thyroid Hormone in Cardiac Dysfunction in Diabetes:

Diabetes is often associated with decreased serum indices of thyroid hormone action in clinical (Saunders et al., 1978) and experimental (Gavin et al., 1981) settings. Many biochemical systems in the myocardium can be affected both by diabetes and hypothyroidism. Sarcolemmal Na-K ATPase (Ku and Sellers, 1982; Chadhury et al., 1987), beta-adrenergic receptor-adenylate cyclase (Ishac et al., 1983; Sundaresen et al., 1984), sarcoplasmic reticulum Ca^{2+} ATPase (Dillmann et al., 1987; Lopaschuk et al., 1983; Limas, 1978), mitochondrial adenine nucleotide translocase (Paulson and Shug, 1984; Mowbray and Corrigal, 1984) and myosin ATPase (Dillmann, 1980; Lompre et al., 1984) all demonstrate reduced activity under both conditions. In many cases activity may be restored by T3 or insulin injection, often by directly increasing the levels of the respective mRNA coding for the enzyme (Dillmann et al., 1987; Chadhury et al., 1987). T3 replacement can partially reverse some of the enzymatic changes in diabetic rats (Dillmann, 1982; Sundaresen et al., 1984). Cardiac function is improved in diabetic rats by T3 administration, but cannot be normalized by T3, even when pharmacologic doses are used (Tahiliani and McNeill, 1985; Garber et al., 1983; Barbee et al., 1988). Additional intervention using the CPT I inhibitor methylpalmoxirate to improve metabolic defects is required to normalize cardiac function in these animals (Tahiliani and McNeill, 1985). Thus, it appears that improvement of fatty acid abnormalities is required in addition to T3 treatment to normalize the heart in diabetes.

Thyroid hormone effects on cardiac contractility at the biochemical level can be explained mainly by its effect of increasing alpha-MHC and decreasing beta-MHC levels, thus increasing %V1 and decreasing %V3 myosin. T3 appears to directly regulate transcription rate of each of the MHC genes by interaction of the T3-nuclear T3 receptor complex with regulatory sequences of the MHC genes. For instance, T3 has a rapid effect on MHC-mRNA levels (Lompre et al., 1984; Dillman, 1984; Gustafsson et al., 1986) and increases transcription rate of the alpha-MHC gene (Darling et al., 1985). T3 can also regulate the expression of a transfected alpha-MHC fusion gene in fetal heart cell culture (Gustafson et al., 1987), and can regulate myosin isozyme expression in the heterotopically isotransplanted heart which does not respond to the work-mediated effects of T3 (Korecky et al., 1987). In addition, thyroid hormone responsive elements similar to those observed for other genes which are regulated by the T3-receptor complex, have been demonstrated in the 5' regulatory sequence of the alpha-MHC gene (Markham et al., 1987).

A reduction in thyroid hormone levels cannot fully account for the switch in MHC isoform expression in diabetic rat heart. The interaction of T3 with a distinct carbohydrate-generated metabolic signal is suggested by the following evidence: 1) a pharmacologic T3 dose is required to normalize cardiac myosin isozyme patterns in diabetic rats (Dillmann, 1982; Garber et al., 1983); 2) the genetically diabetic BB/W rat develops the typical shift in myosin isozymes without reduced thyroid hormone levels (Malhotra et al., 1985); and 3) chronic administration of the CPT I inhibitor

methylpalmoxirate to diabetic rats can reverse the diabetic pattern of myosin isozyme expression (Dillmann, 1985). Unfortunately, in this last case it was not determined exactly what metabolic effects inhibition of fatty acid oxidation had in conjunction with the observed myosin isozyme changes. In the liver, a similar interaction between carbohydrate and T3 signals has been observed for the induction of several proteins related to lipogenesis (Mariash and Oppenheimer, 1985). It was shown that DCA, which increases PDH activity by inhibiting PDH kinase, could mimic the effects of glucose in inducing malic enzyme in cultured hepatocytes, even in the presence of glucagon. T3 was suggested to amplify the carbohydrate signal at a post-T3 receptor site, based on analysis of T3 receptor occupancy and the rapid synergistic effects of the two agents in increasing specific mRNA levels (Mariash and Oppenheimer, 1985; Mariash et al., 1986).

Purpose of Investigations:

The focus of these experiments was the role of the glucose-fatty acid cycle in causing disturbances of cardiac function and glucose oxidation in diabetes. Dillmann (1985) has demonstrated that chronic administration of methylpalmoxirate, which inhibits long chain fatty acid oxidation by blocking CPT I, could partially reverse the shift in cardiac myosin isozyme predominance in diabetic rats. These results suggested that increased glucose oxidation as a result of CPT I inhibition signalled the myosin isozyme transitions, since serum thyroid hormone levels were unchanged. Neither the associated improvements in carbohydrate metabolism nor the effects on cardiac

function were investigated in this study. The purpose of the first study was to determine if reactivation of the PDH complex could increase myocardial glucose oxidation rates, myofibril ATPase, and cardiac function in diabetic rats. DCA was administered to chronically diabetic rats for 5 weeks in order to directly activate the PDH complex. This duration of treatment was chosen since the switch in myosin isozyme pattern in diabetes is completed after at least 4 weeks. Glucose oxidation rates and cardiac function were measured using the isolated working heart apparatus in presence of a high concentration of fatty acids, in order to approximate the diabetic condition. Myofibril ATPase activity was determined in tissue from these same hearts frozen after perfusion.

Glucose oxidation rates are profoundly suppressed in the presence of fatty acids. Serum and myocardial levels of fatty acids are elevated in diabetes. Fatty acids may also play a role in decreased cardiac function in diabetes. The short-term, reversible effects of fatty acids on myocardial glucose oxidation and cardiac function in diabetes had not been investigated. The purpose of the second study was therefore to determine the acute effects of fatty acids and of CPT I inhibition on myocardial glucose oxidation rates and cardiac function in chronically diabetic rats. Hearts were perfused in the isolated working heart apparatus with buffer containing glucose in the absence and presence of a high fatty acid concentration. The phenylalkyloxirane carboxylic acid, Etomoxir, was also added to the perfusate to determine if CPT I inhibition could reverse the effects of fatty acids.

Experimental Models:

The isolated working rat heart is a good model for the study of cardiac metabolism and function. The contribution of carbohydrate and lipid substrates to energy metabolism closely parallels that seen in the human (Wisneski et al., 1987; Neely and Morgan, 1974). While neural influences which would be seen in vivo are absent, this factor along with flow, oxygenation, work, substrate, and hormonal conditions can be controlled in the isolated working heart. Cardiac function can be assessed by measuring heart rate, peak systolic pressure, and/or cardiac output. In the present study, the rate-pressure product (HRXPSP) was chosen as an indicator of cardiac function. This has been extensively used in the past as to assess cardiac function in normal and diabetic rat hearts (see for example, Garber and Neely, 1983), and has been shown to be highly correlated to indices of performance of the heart such as cardiac output in our laboratory, under such conditions as diabetes and ischemia. Rate-pressure product is also a good indicator of oxygen consumption or metabolic rate of the heart and can therefore be used to compare substrate oxidation rates relative to the total energy demand of the heart

The streptozotocin diabetic rat is a non-insulin dependent model of diabetes which has been well-characterized by previous investigators. The genetically diabetic "BB" Wistar rat is probably more similar to the human insulin-dependent diabetic in that insulin injections are required for survival. Metabolism of the diabetic "BB" Wistar rat heart has not been extensively studied and therefore, comparisons between this model and the alloxan or streptozotocin

models of diabetes would be incomplete at this time. A study by Lopaschuk and Tsang (1987) suggests that insulin withdrawal from diabetic "BB" Wistar rats for as little as 24 hours can lead to metabolic disturbances such as increased myocardial lipid storage, and decreased heart rate. Similar to streptozotocin diabetes in the rat, serum lipid and and glucose profiles can be disturbed in human insulin-dependent diabetics, and this is related to the degree of insulin deficiency (Denton and Randle, 1967; Taylor and Agius, 1988). The decreased heart rate, contractility, and altered myosin isozyme pattern seen in chemically induced diabetic rat hearts is also seen in the diabetic "BB" Wistar rat. However, in human heart, the fast V1 myosin ATPase does not exist. Therefore, it is unknown whether metabolic disturbances would affect contractility of the human heart by inducing a slower myosin isozyme. For obvious reasons, the defects in other enzyme systems such as sarcoplasmic reticulum Ca^{2+}ATPase have not been examined in hearts from human diabetics. The diabetic rat heart is also a good model to examine the direct metabolic effects of diabetes on heart function, independent of possible increased incidence of coronary artery disease in diabetes, since diabetic rats do not develop this problem (Gotzsche, 1986).

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CHAPTER 2: DICHLOROACETATE INCREASES GLUCOSE OXIDATION AND MYOFIBRIL ATPase IN DIABETIC RAT HEART*

ABSTRACT

Previous reports have suggested that administration of agents which reactivate pyruvate dehydrogenase (PDH) can increase cardiac myosin ATPase activity by increasing the percent of myosin as the V1 isozyme and decreasing the percent as the V3 isozyme. We therefore studied the effect of chronic administration of dichloroacetate (DCA), which directly activates PDH, on glucose oxidation rates and myofibril ATPase activity in diabetic rat hearts. Control and 4 week streptozotocin diabetic rats were administered DCA (2 mg.(g body weight)$^{-1}$) in their diet for 5 weeks. Glucose oxidation rates were subsequently measured as $^{14}CO_2$ production from ^{14}C-glucose (11 mM) in isolated working hearts in the presence of fatty acids (1.2 mM palmitate). Glucose oxidation was markedly decreased in diabetic versus control rat hearts (18$\pm$3 and 204$\pm$44 nmol. g dry^{-1}.min^{-1}, respectively). Both maximal Ca^{2+}-activated cardiac myofibril ATPase activity and

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heart rate were also depressed in diabetic compared to control rats. DCA treatment significantly increased glucose oxidation rates in diabetic rat hearts (from 18±3 to 85±13 nmol. g dry^{-1}. min^{-1}), and prevented the decrease in maximal Ca^{2+}-activated myofibril ATPase activity from occurring in diabetic rats. However, heart function (HRXPSP product) was not improved in diabetic rats treated with DCA. Depressed serum thyroxine in diabetic rats was unaffected by DCA treatment. These data suggest that a metabolic signal distinct from thyroid hormones can regulate cardiac contractile protein function, probably at the level of myosin isozyme expression. Other factors must be responsible for the persisting cardiac dysfunction in isolated working hearts from diabetic rats.

INTRODUCTION

Increasing evidence suggests that a cardiomyopathy independent of atheroschlerotic lesions develops in humans and animals with diabetes mellitus. Epidemiologic and clinical studies indicate an impaired cardiac function in diabetics which is related to the metabolic disturbances of the disease (Fisher et al., 1987; Kannel and McGee, 1979). A depression of cardiac function developing over several weeks has also been demonstrated in experimental models of diabetes such as the rat (Ingebretson et al., 1980; Fein et al., 1980; Miller, 1979; Penparkgul et al., 1980; Vadlamudi et al., 1982). These chronic changes are associated with changes in cardiac membrane function, such as reduced Ca2+ transporting ability of the sarcoplasmic reticulum (Ganguly et al., 1983; Lopaschuk et al., 1983; Penparkgul et al., 1981). A decrease in myofibril and myosin ATPase

activity also develops in the heart (Dillman, 1980; MacLean et al., 1987; Malhotra et al., 1981; Pierce and Dhalla, 1981).

Rat cardiac myosin exists as three isoforms with varying ATPase activity. These isoforms are distinguished by the types of myosin heavy chain (MHC) that form the MHC dimer in the myosin molecule,i.e., V3-$\beta\beta$MHC homodimer; V2-$\alpha\beta$MHC heterodimer; V1-$\alpha\alpha$MHC homodimer) (Hoh et al., 1978, 1979). An increase in myosin ATPase activity as the isoform predominance shifts from V3-V1 myosin is thought to be correlated to mechanical indices of contractility (Ebrecht et al., 1982; ; Lecarpentier et al., 1987; Pagani and Julian, 1984; Rupp, 1982; Schwartz et al., 1981). Chronic diabetes or hypothyroidism in the rat induces a shift in myosin isoform predominance from V1 to V3 (Dillmann, 1980; Hoh et al., 1978). Triiodothyronine (T3) induces αMHC expression and suppresses βMHC expression by transcriptional regulation of the genes for each of these proteins (Gustafson et al., 1987; Lompre et al., 1984; Mahdavi et al., 1984), thereby increasing V1 and decreasing V3 myosin predominance. While diabetes is accompanied by decreased serum levels of thyroid hormones, the fact that there is a distinct carbohydrate-related metabolic signal to MHC expression is suggested by several lines of evidence. Firstly, a pharmacologic T3 dose is needed to normalize cardiac myosin ATPase activity and myosin isozyme pattern in diabetic rats (Dillman, 1982; Garber et al., 1983). Secondly, the genetically diabetic "BB/W" rat develops the typical shift in myosin isozyme pattern without reduced thyroid hormone levels (Malhotra et al., 1985). Finally, administration of methylpalmoxirate (an inhibitor of fatty acid oxidation) to diabetic

rats reverses myosin isozyme changes without increasing thyroid hormone levels (Dillman, 1985). At a functional level, it has been demonstrated that performance of isolated working hearts from diabetic rats can be normalized by administration of T3 and methylpalmoxirate, but not T3 alone (Tahiliani and McNeill, 1984).

In diabetic compared with normal hearts, a greater proportion of myocardial energy provision is derived from oxidation of lipid fuels (Denton and Randle, 1967). Apart from the primary defect, insulin lack, elevated levels of circulating fatty acids and increased myocardial triacylglycerol stores are thought to contribute to a decrease in glucose oxidation rates. This is thought to occur through accumulation of mitochondrial intermediates of fatty acid oxidation such as citrate and acetyl CoA, which inhibit phosphofructokinase 1 (PFK 1) and pyruvate dehydrogenase (PDH), respectively (Randle et al., 1963; Randle et al., 1984). Thus, perfusion with agents that either reactivate the PDH complex or inhibit fatty acid transport into the mitochondrion can greatly increase glucose oxidation rates in the diabetic rat heart. Both dichloroacetate (DCA), which activates the PDH complex by inhibiting PDH kinase, and carnitine palmitoyltransferase I (CPT I) inhibitors such as the phenylalkyloxirane carboxylic acids POCA and Etomoxir are agents which have been shown to increase glucose oxidation in diabetic rat hearts (McAllister et al., 1973; Rosen and Reinauer, 1984; Wall and Lopaschuk, 1988).

Chronic administration of the CPT I inhibitor methylpalmoxirate, to diabetic rats has been shown to partially

reverse the shift in myosin isozyme pattern (Dillman, 1985). However, it was not determined whether PDH activity, glucose oxidation rates, or mechanical function were concommitantly increased in hearts from these diabetic rats. Tahiliani and McNeill (1985) were unable to demonstrate an improvement in cardiac performance in diabetic rats administered methylpalmoxirate. The purpose of this study was to determine if improvement of myocardial glucose oxidation is associated with both reversal of depressed cardiac myofibril ATPase and function in the diabetic. To do this, DCA was administered to chronically diabetic rats over a period of 5 weeks to overcome the suppressed PDH activity seen in diabetes. Myofibril ATPase activity, glucose oxidation rates, and mechanical function were subsequently measured in fatty acid-perfused isolated working hearts from these animals.

MATERIALS AND METHODS

Streptozotocin was obtained from Upjohn. DCA was purchased from BDH Chemicals. Bovine serum albumin (fraction V) was purchased from Sigma. D-[U-^{14}C]-glucose (1-5 mCi.mmol^{-1}) was purchased from New England Nuclear. Palmitic acid was a product of Kodak. Insulin was obtained from Connaught Novo Laboratories (Toronto). Thyroxine radioimmunoassay kits were purchased from Radioassay Systems Laboratories (Carson, California). Quality control sera were from Biomega Diagnostics (Montreal). All other chemicals were reagent grade.

Animals:

Male Sprague Dawley rats (200-250 grams) were anesthetized

with halothane and injected with 65 $mg.kg^{-1}$ streptozotocin dissolved in citrate buffer (pH 4.5) into the dorsal penile vein. Control animals were injected with the same volume of citrate buffer only. Serum glucose was determined using a Beckman Glucose Analyzer 2. Rats with a serum glucose greater than 350 $mg.dl^{-1}$ were considered diabetic. Animals were allowed water ad libitum and were maintained on a reverse day-night cycle in hanging metabolic cages.

Animals were fed standard rat chow. Food consumption was monitored by periodic weighing of ground chow contained in feeding dishes that allowed free access to food without spillage. At 4 weeks, serum glucose was again determined and the control and diabetic groups were then randomly divided into 2 control groups and 2 diabetic groups. One control and one diabetic group was fed normal chow and the other 2 groups were fed the same diet but with DCA added. DCA was first neutralized, then dried onto lab chow, and administered at a dose of 2 mg DCA.$(g\ body\ weight.day)^{-1}$ was administered. This was achieved by continuous monitoring of food intake and body weight, and individual adjustment of DCA concentrations in the food. This second phase of the experiment lasted 5 weeks, during which time serum glucose was determined after 2 and 4 weeks.

Heart Perfusions:

Hearts from Na pentobarbitol anesthetized rats were excised and cannulated as described previously (Lopaschuk et al., 1986). Blood was removed from the thoracic cavity for determination of serum glucose and thyroxine. Hearts were initially perfused retrogradely through the aorta for 10 minutes with Krebs-Henseliet buffer, pH 7.4,

gassed with 95% O_2, 5% CO_2, containing 2.5 mM free calcium and 11 mM glucose. During this time the left atrium was cannulated. Hearts were then switched to the working heart mode and perfused with buffers containing 11 mM ^{14}C-glucose (600,000 dpm.ml^{-1}) and 3% bovine serum albumin. Depending on the experiment, 1.2 mM palmitate, 0.4 mM palmitate plus 20 μU.ml^{-1} insulin, or 500 μU.ml^{-1} insulin, was also added to the perfusate. When added, palmitate was prebound to albumin. Unless otherwise stated, hearts were perfused at a left atrial filling pressure of 15 cm H_2O and hydrostatic aortic afterload of 80 mm Hg. Glucose oxidation was measured as steady state $^{14}CO_2$ production from ^{14}C-glucose, as described previously (Lopaschuk et al., 1986). Briefly, at 10 minute intervals, $^{14}CO_2$ was collected from a closed recirculating system connected to a methylbenzethonium hydroxide CO_2 trap (for gaseous $^{14}CO_2$) and from buffer samples via a sampling port (for ^{14}C-bicarbonate). Buffer samples were injected into closed metabolic flasks containing H_2SO_4 to drive off $^{14}CO_2$ which was collected in centrewells containing methylbenzethonium hydroxide. At the end of perfusion, hearts were freeze-clamped with Wollenberger clamps cooled in liquid N_2.

Myofibril ATPase:

Cardiac myofibrils were isolated from frozen hearts by differential centrifugation and assayed for myofibril ATPase activity as described by MacLean et al.(1987). Freshly isolated myofibrils (at a final protein concentration of 0.25 mg.ml^{-1}) were initially preincubated at 30 degrees C in a 1 ml reaction medium containing 5

mM Tris(hydroxymethyl)aminomethane (pH 7.0), 75 mM KCl, 1 mM $MgCl_2$ and various free Ca^{2+} concentrations as calculated by MacLean et al. (1987). ATPase activity was determined by measuring P_i accumulation over a 5 minute period following addition of MgATP to a final concentration of 5 mM. The reaction has been demonstrated to be linear over this time period. The reaction was terminated by addition of 1 ml of cold 20% trichloroacetic acid. Tubes were centrifuged at 1000 g for 10 minutes and inorganic phosphate was determined on the protein-free supernatant. Values were corrected for non-specific ATP hydrolysis by addition of trichloroacetic acid prior to ATP. Contamination from mitochondrial ATPase was negligible, as determined by Na azide sensitivity. Basal (Mg^{2+}-stimulated) ATPase activity was measured in the presence of 5 mM EGTA. Ca^{2+}-stimulated ATPase activity was determined by subtracting basal from total (Mg^{2+}+Ca^{2+}) activity. pCa_{50} was determined as Ca^{2+} concentration at half maximal ATPase activity from curve-fitted Ca^{2+}-activation plots using the computer program GRAPHPAC on an Apple computer.

Data were analyzed using two-way ANOVA followed by the Newman Keuls test or Student's t-test, using a significance level of $p < 0.05$.

RESULTS

DCA treatment was initiated at 4 weeks following the onset of diabetes. The amount of DCA ingested during the 5 week treatment period averaged from 1.41-2.62 mg.(g body weight.day)$^{-1}$ and was similar in diabetics and controls. Physiologic variables of rats in

the 4 groups at the end of the study are shown in Table 1. Diabetic animals had reduced serum T4 levels compared to control rats. Ventricular weight was reduced in DCA-fed rats compared with untreated controls, and also in DCA-treated controls versus untreated diabetics. Myofibril protein yield was decreased in DCA-treated diabetics versus untreated controls. Elevated serum glucose levels in diabetic animals persisted throughout the study period in untreated and DCA-treated rats. DCA-treated rats, however, did have significantly lower serum glucose levels at 6 and 8 weeks of diabetes.

Figure 1 shows the food intake of rats throughout the experimental treatment period. As expected, untreated diabetic animals were hyperphagic compared with controls. DCA treatment resulted in a decrease in food intake per gram body weight in diabetic and control animals compared to their respective control groups. Figure 2 shows the body weights of the rats throught the study. As expected, weight gain in diabetic rats was reduced compared to control animals. DCA treated diabetic and control animals showed a decrease in body weight gain compared to their respective controls. The decrease in food intake in both DCA-fed control and diabetic rats (see Figure 1) appears to be responsible for decreased growth rate in these rats.

Initial experiments were performed in control rat hearts to determine the effects of fatty acids, insulin, and increased cardiac work would have on glucose oxidation rates (Figure 3). Addition of fatty acids to the perfusate at levels which can be observed in diabetes (1.2 mM palmitate), results in a substantial drop in glucose

oxidation to about 20% of rates seen in glucose-perfused hearts (204±44 nmol.g $dry^{-1}.min^{-1}$). In hearts perfused with buffer approximating normal fatty acid and insulin levels (0.4 mM palmitate + 20uU/ml insulin), glucose oxidation rates are 640±167 nmol.g $dry^{-1}.min^{-1}$ (Figure 3), Addition of high levels of insulin to normal hearts perfused with glucose alone approximately doubles glucose oxidation. Subsequently increasing cardiac work in these hearts by increasing left atrial filling pressure and hydrostatic afterload raises glucose oxidation rates to over 3000 nmol. g $dry^{-1}.min^{-1}$. Since fatty acid levels are elevated in diabetes, and serum insulin levels are decreased, all subsequent glucose oxidation rates were measured in the presence of 1.2 mM palmitate. To ensure that DCA directly increases myocardial glucose oxidation under these conditions, experiments were also performed where DCA (0.5-5 mM) was added directly to the perfusate of isolated working hearts. A marked increase in glucose oxidation was seen in palmitate-perfused hearts from chronically diabetic rats (from 34±3 to 968±130 nmol. g $dry^{-1}.min^{-1}$).

Glucose oxidation rates in hearts from control and diabetic rats fed normal chow or the DCA diet are shown in Figure 4. Compared to controls, glucose oxidation was greatly reduced in diabetic rat hearts. DCA treatment of control rats decreased glucose oxidation rates, although this was not significant. However, in diabetic animals DCA treatment resulted in an increase in glucose oxidation.

Figure 5 shows Ca^{2+}-activation curves for Mg^{2+}-stimulated myofibril ATPase in the 4 experimental groups determined at the end of the experimental protocol. (Ca^{2+}+Mg^{2+})

myofibril ATPase was significantly reduced at pCa^{2+} 5.3 and 5 in diabetics compared with all other groups. DCA treatment normalized maximal Ca^{2+}-activated, Mg^{2+}-stimulated myofibril ATPase activity in diabetic rats, and was without effect on myofibril ATPase in control rats. Table 2 shows basal (Mg^{2+}-stimulated) myofibril ATPase activities and pCa_{50} values measured in the study. Basal ATPase activity was reduced in untreated diabetics compared to controls, and was not significantly increased by DCA-treatment in diabetic rats. There were no differences in Ca^{2+}-stimulated ATPase activity at any pCa^{2+} between the 4 experimental groups (data not shown), suggesting unchanged calcium sensitivity of myofibril ATPase. Calculated pCa_{50} values were no different among the groups.

Heart function (HRXPSP) was also determined in DCA-treated and untreated control and diabetic rats (Table 3). Heart rate was significantly reduced in untreated diabetics versus controls, although the HRXPSP product was not different between the groups. Both heart rate and HRXPSP were reduced in DCA-treated diabetics compared to untreated controls. In addition, DCA treatment also resulted in decreased heart function in control animals.

DISCUSSION

Results from this study show that depressed cardiac myofibril ATPase activity in diabetic rats can be normalized by chronic administration of DCA, an agent which activates the PDH complex. Suppression of the PDH is thought to play a key regulatory role in depressing myocardial glucose oxidation in diabetes (Randle

et al., 1984). When glucose oxidation rates were measured in isolated working hearts from DCA-treated animals, a dramatic increase was seen. These data therefore support the concept that a metabolic signal related to myocardial glucose oxidation regulates function of the cardiac contractile proteins. Since myofibril ATPase activity is correlated with myosin isoform predominance (Rupp,1982; Ebrecht et al., 1982), it seems likely that the increased myofibril ATPase activity in diabetic rats after DCA treatment is due to altered rates of expression of the two MHC genes which determine the myosin isozyme pattern. This is supported by the work of Dillman (1984, 1985) who demonstrated increased cardiac myosin ATPase activity and increased %V1 myosin in diabetic rats after experimental treatments designed to increase myocardial carbohydrate utilization. The experimental protocol used in his studies, however, did not include an assessment of heart function. In our study, although myofibril ATPase activity was normalized by DCA treatment in diabetic rats, we failed to see an improvement in heart function in isolated working hearts from DCA-fed diabetic rats. These data indicate that factors other than decreased myofibril ATPase activity must be contributing to reduced rate-pressure product in diabetic rat hearts.

The involvement of carbohydrate utilization and thyroid hormone signals in the regulation of rat cardiac MHC genes is evident from observations that a high carbohydrate or fructose diet can induce V1 myosin in hypothyroid rats (Dillman, 1985) and that T3 can increase %V1 myosin in diabetic rat hearts (Dillman, 1982). In addition, Mariash and Oppenheimer (1985) have shown that some hepatic proteins involved in lipogenesis and the pentose phosphate pathway

are under similar dual regulation by carbohydrates and T3. They demonstrated that DCA could mimic the effects of glucose on induction of malic enzyme in hepatocytes, even in the presence of glucagon. They suggested that the observed synergistic induction of proteins such as malic enzyme by the two signals was due to a multiplication of the carbohydrate signal by thyroid hormone (Mariash and Oppenheimer, 1985, Mariash et al., 1986). In heart, Dillman (1985) reported increased cardiac αV1 myosin without increase in serum thyroid hormone levels in diabetic rats after feeding the CPT I inhibitor, methylpalmoxirate, which would theoretically increase myocardial glucose oxidation by activating PDH. In our study, we directly measured the effects of DCA treatment on glucose oxidation and on contractile protein biochemistry in the same hearts. Our data support the conclusion of Dillmann, and demonstrate an increase in glucose oxidation with a concommitant increase in myofibril ATPase activity. In addition, since DCA is does not directly inhibit fatty acid flux, this suggests that the effects of methylpalmoxirate on myosin ATPase observed by Dillmann are due to an inhibition of fatty acid oxidation which allows an increase in glucose oxidation.

Myocardial glucose oxidation is highly dependent on the presence of fatty acids and/or insulin in the perfusate, as well as the rate of work performed by the heart. As shown in Figure 3, addition of palmitate to glucose-perfused control hearts reduced glucose oxidation to rates seen in glucose-perfused diabetic rat hearts (Wall and Lopaschuk, 1988; Garland et al., 1964), demonstrating the key role of fatty acids in inhibiting myocardial glucose oxidation. However, other factors are also involved in

decreasing glucose oxidation in diabetes. Suppression of glucose oxidation due to inactivation of the PDH complex is shown by resistance of PDH to activation by the CPT I inhibitor tetradecylglycidic acid in the perfused diabetic rat heart (Caterson et al., 1982). PDH is also resistant to such activators as Ca^{2+} and DCA in isolated mitochondria from diabetic rat hearts (Kerbey et al., 1985). The percentage of PDH in the active form (%PDHa) is reduced in diabetes by increased PDH kinase activity, both intrinsic to the complex and in the form of a free "kinase/activator" protein, resulting in increased phosphorylation of the complex and resistance to PDH phosphatase (Randle et al., 1984; Kerbey et al., 1984, 1985). The hormonal and/or metabolic factors which cause this stable reduction in %PDHa in diabetes are unknown but are probably related to chronically increased oxidation of lipid fuels (Randle et al., 1984; Fatania et al., 1986). The net effect is that glucose oxidation is almost nonexistent in diabetic rat hearts perfused with palmitate at a concentration approximating in vivo levels (see Figures 3 and 4).

DCA increases %PDHa in the heart by inhibiting PDH kinase (Whitehouse et al., 1974). Addition of millimolar concentrations of DCA to the perfusate can increase glucose oxidation rates in normal and diabetic rat hearts perfused with glucose and insulin, as well as in hearts from normal or starved rat hearts in the presence of added acetate, ketone bodies, or palmitate (McAllister et al., 1973; Higgins et al., 1978). We show that chronic DCA treatment could increase glucose oxidation rates in diabetic rat hearts even in the presence of a high concentration of fatty acids. One problem

encountered with the DCA treatment regime was a decreased food intake in rats fed DCA. However, this cannot account for the observed effects of DCA, since the resultant increased serum fatty acid levels with reduced food intake would produce a decrease in glucose oxidation. The effect of DCA on glucose oxidation was further confirmed by experiments in which DCA was added directly to the perfusate of isolated working hearts. In untreated diabetic rat hearts perfused with palmitate, DCA eliminated the effect of *in vitro* addition of palmitate and of the diabetic condition on glucose oxidation. Thus, pyruvate must have effectively competed with fatty acids for oxidation in the mitochondrion, as suggested by earlier work (Brosnan and Reid, 1986; McAllister et al., 1973; Forsey et al., 1987; Higgins et al., 1978). These results demonstrate the key role of the PDH complex in regulation of myocardial glucose oxidation by fatty acids and in diabetes.

Contractile performance of isolated working hearts perfused with glucose is reduced in diabetic rats. As demonstrated in this study, cardiac function is also depressed in the presence of fatty acids. Depressed function in long-term diabetes is thought to be due to slowly developing defects in membrane function and decreased Ca^{2+}-activated myosin ATPase activity and altered myosin isozyme distribution. In accord with previous observations (Pierce and Dhalla, 1981; MacLean et al., 1987), we also observed depressed myofibril ATPase activity in diabetic rat hearts. Although myofibril ATPase activity was normalized by DCA treatment in the diabetics, heart function at this workload was not improved in these animals (see Table 3). A dissociation between contractile protein

biochemical activity and mechanical function has also been demonstrated in other studies. Popovich et al. (1987) have shown that in diabetic rats administered methylpalmoxirate, mechanical Vmax of isolated papillary muscles was unchanged even though myosin ATPase and %V1 myosin were increased compared to untreated diabetic rats. A T3 replacement dose which normalizes myosin ATPase activity does not prevent the decline in left ventricular pressure development in diabetic rats (Garber et al., 1983; Tahiliani and McNeill, 1985). Together, these data indicate that changes in myosin ATPase activity do not necessitate changes in in cardiac function, as indicated by rate-pressure product.

In summary, we have shown that reduced glucose oxidation rates and myofibril ATPase activity in diabetic rat heart can be simultaneously improved by chronic administration of DCA. This supports previous work suggesting that inactivation of the PDH complex is an important mechanism of reduced myocardial glucose oxidation in diabetes. The increase in Ca^{2+}-activated myofibril ATPase in DCA-treated diabetic animals occurred without change in serum thyroid hormone levels, suggesting that alterations in metabolic rates of glucose or fatty acids per se signal changes in contractile protein function. Together, these data suggest that a common metabolic signal related to energy metabolism may regulate myosin isozyme expression. The nature of this putative metabolic signal and the mechanism by which it may interact with thyroid hormone in regulating expression of myocardial proteins such as myosin are important questions which remain to be elucidated.

Table 1: Physiologic characteristics of control and diabetic rats treated with dichloroacetate.

	CONTROL		DIABETIC	
	Untreated	DCA-Fed	Untreated	DCA-Fed
Ventricle Wet Weight (grams)	1.67±0.05	1.19±0.04*t	1.45±0.13	1.19±0.13*
Ventricle/ Body Weight Ratio(X1000)	3.45±0.12	3.91±0.08*	3.98±0.35	4.07±0.16
Myofibril Protein Yield (mg/g)	30.35±2.45	25.77±1.78	27.90±0.87	20.96±2.29*
Serum T4 ($ug.dL^{-1}$)	4.16±0.18	1.59±0.30*	2.25±0.14*	1.26±0.36*
Serum Glucose (mM)				
4 day	-	-	32.48±3.01	30.53±1.21
4 week	-	-	29.20±2.46	27.36±1.70
6 week	-	-	37.38±1.91	24.40±1.96t
8 week	-	-	36.93±1.69	21.64±2.71t

Unless otherwise indicated, data was obtained from rats at the end of the 9 week experimental treatment period. Data are mean±SEM from at least 5 animals per group. *, significantly different from untreated control; t, significantly different from untreasted diabetic.

Table 2: Myofibril ATPase activity of ventricles from dichloroacetate-treated control and diabetic rats.

	CONTROL		DIABETIC	
	Untreated	DCA-Fed	Untreated	DCA-Fed
Basal (Mg^{2+}-stimulated) myofibril ATPase (umol P_i. mg^{-1}. min^{-1})	0.0578 ±0.007	0.0665 ±0.012	0.0300 ±0.010*	0.0465 ±0.005a
pCa_{50}	6.085 ±0.099	6.315 ±0.172	6.254 ±0.082	6.142 ±0.037

Hearts are the same as those described in Table 3. Basal (Mg^{2+}-stimulated ATPase activity was determined in the presence of EGTA as described in Methods). pCa_{50} is the Ca^{2+} concentration at half-maximal (Ca^{2+}+Mg^{2+}) ATPase activity for each heart. *, significantly different from untreated control. a, significantly different from DCA-fed control.

Table 3: Function of isolated working rat hearts from dichloroacetate-treated control and diabetic rats.

	CONTROL		DIABETIC	
	Untreated	DCA-Fed	Untreated	DCA-Fed
Heart Rate (beats.min^{-1})	226.5±13.2	166.3±7.2*	183.0±19.7*	168.3±8.6*
Peak Systolic Pressure (mm Hg)	112.1±6.9	100.1±11.8	122.6±6.9	115.8±4.2
HRXPSPX10^{-3} (mm Hg.beats. min^{-1})	25.35±1.86	16.50±1.54*	21.94±1.42	19.43±1.00*

Hearts are the same as those described in Figure 4. *, significantly different from untreated controls.

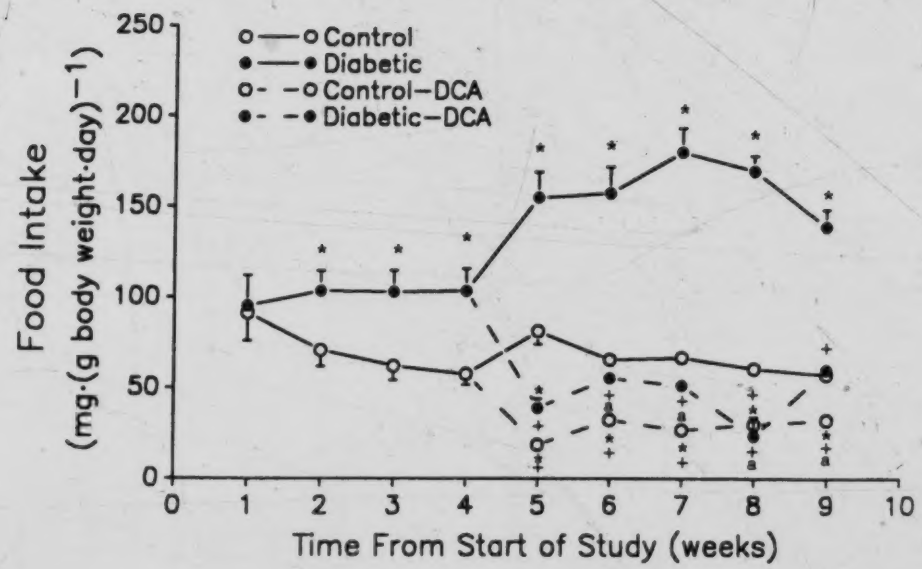

Figure 1: Food intake of dichloroacetate-treated control and diabetic rats.

Food intake was measured as described in Methods and is corrected for individual body weight. Values are mean±SEM for 5 animals. *, $p<0.05$ versus control, normal chow; +, $p<0.05$ versus diabetic, normal chow; a, $p<0.05$ versus diabetic-DCA.

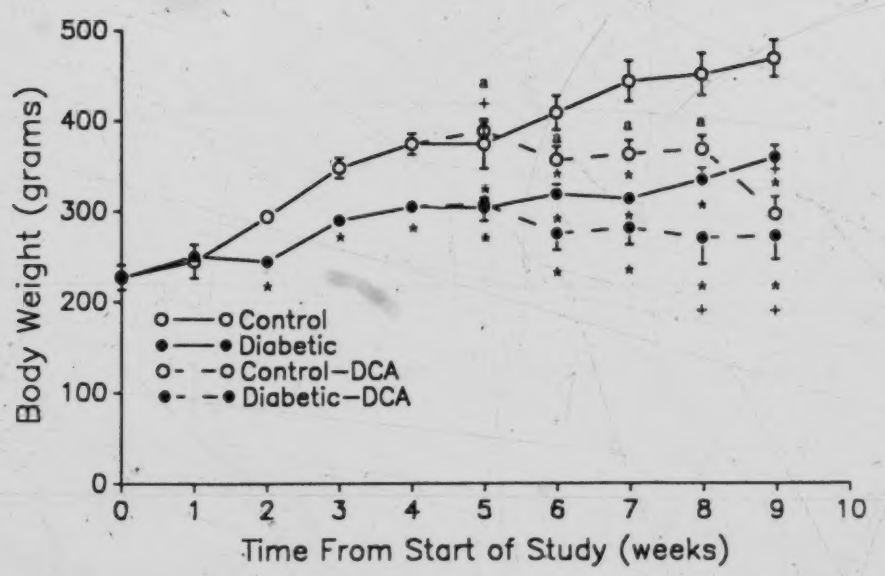

Figure 2: Body weight of dichloroacetate-treated control and diabetic rats.

Rats are the same as described in Figure 1. *, significantly different from untreated control. +, significantly different from untreated diabetic. a, significantly different from DCA-fed diabetic.

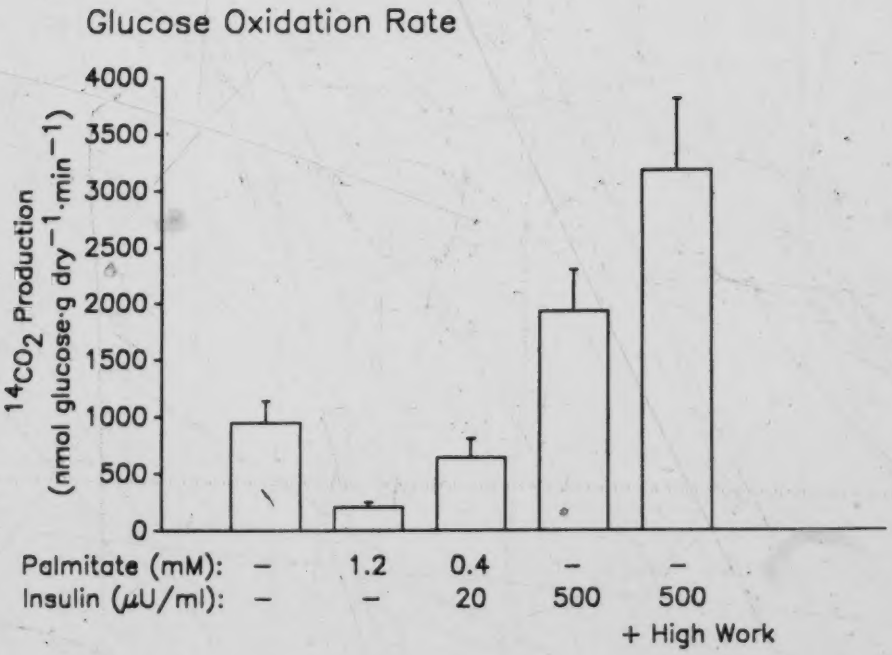

Figure 3: Effects of insulin, palmitate, and increased work on glucose oxidation rates in normal rat hearts.

Glucose oxidation rates were determined as described in Methods in the presence of 11 mM ^{14}C-glucose and other additions as indicated. In the high cardiac work situation, left atrial filling pressure was increased from 15 to 25 cm H_2O and aortic afterload was increased to 150 mm Hg. Values are mean±SEM for at 2-5 hearts.

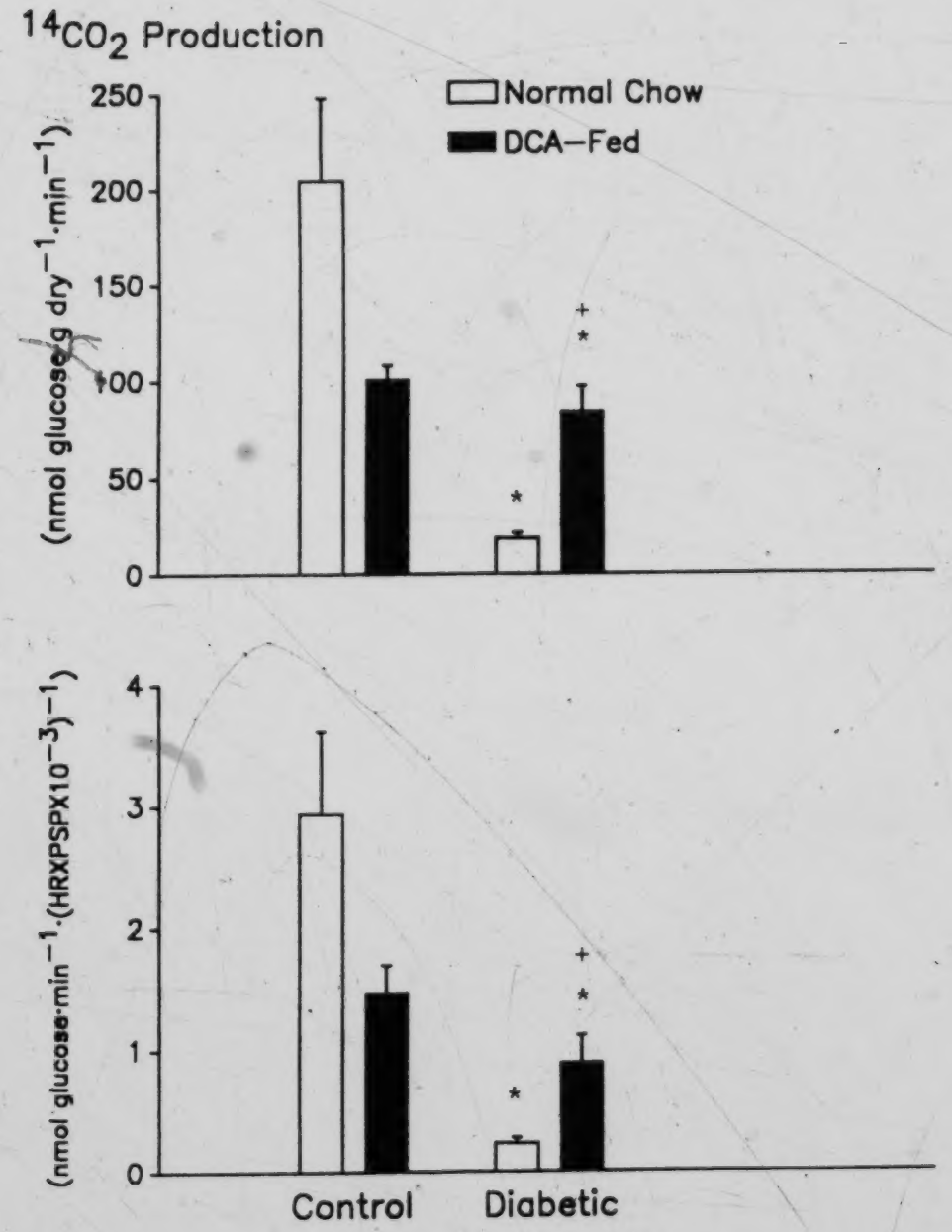

Figure 4: Glucose oxidation rates in dichloroacetate-treated control and diabetic rats.

Hearts were obtained from animals after the 9 week experimental treatment period and were perfused in the presence of 11 mM ^{14}C-glucose and 1.2 mM palmitate. Glucose oxidation rates were measured as described in Methods and were expressed per gram of dry heart weight (A), or per (HRXPSPX10^{-3}) (B). Values are mean±SEM of at 5 hearts in each group. *, significantly different from untreated control. +, significantly different from untreated diabetic.

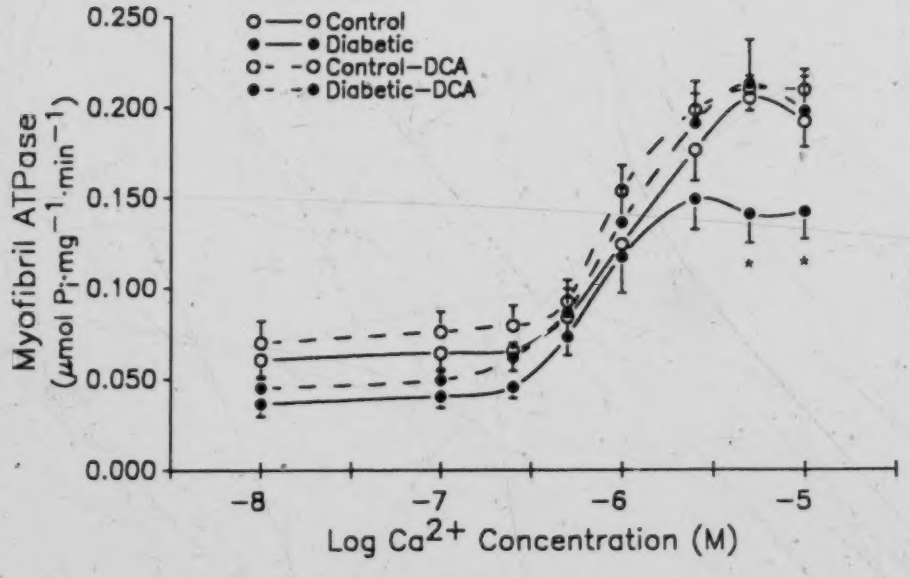

Figure 5: Ca^{2+}-activation plots of cardiac myofibril ATPase in dichloroacetate-treated control and diabetic rats.

Hearts are the same as those described in Figure 4. Mg^{2+}-stimulated myofibril ATPase was determined as increasing free Ca^{2+} concentrations as described in Methods. *, significantly different from other groups.

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CHAPTER 3: GLUCOSE OXIDATION RATES IN FATTY ACID-PERFUSED ISOLATED WORKING HEARTS FROM DIABETIC RATS*

ABSTRACT

The contribution of elevated fatty acid levels to decreased myocardial glucose oxidation in diabetes was studied. $^{14}CO_2$ production from 11 mM ^{14}C-glucose was measured in control or 6 week streptozotocin-diabetic isolated working rat hearts perfused with or without 1.2 mM palmitate (prebound to 3% albumin). In control hearts, addition of palmitate to the buffer resulted in a marked reduction (13-fold) in glucose oxidation rates, to rates seen in diabetic rat hearts perfused in the absence of palmitate. Glucose oxidation in diabetic rat hearts perfused with palmitate was almost completely suppressed. Even though glucose oxidation rates were low, exogenous palmitate oxidation rates, measured as $^{14}CO_2$ production from ^{14}C-palmitate, were not increased in diabetic versus control hearts. Addition of the carnitine palmitoyltransferase I inhibitor, Etomoxir (10^{-6} M), resulted in a doubling of glucose oxidation rates in control rat hearts in the presence or absence of palmitate, as well as in diabetic rat hearts perfused in the absence of

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palmitate. In palmitate-perfused diabetic rat hearts, a relatively smaller, but still significant increase also occurred. The effects of Etomoxir on glucose oxidation could not be explained by reduced exogenous palmitate oxidation, or decreased levels of citrate. Cardiac function, as measured by the heart rate X peak systolic pressure product, was reduced in diabetic rat hearts. Etomoxir significantly increased heart function in palmitate-perfused hearts from both control and diabetic rats. These data suggest that acutely reversible effects of fatty acids can contribute to decreased glucose oxidation and cardiac function in diabetic rat hearts.

INTRODUCTION

Myocardial glucose oxidation is reduced in diabetes mellitus by several mechanisms. The primary defect, insulin lack, results in diminished glucose uptake. In addition, according to the classic concept of the glucose-fatty acid cycle (Randle et al., 1963, 1984), elevated serum and tissue fatty acid levels in diabetes are also associated with decreased glycolytic rates. Increased citrate concentration, which inhibits phosphofructokinase 1 (PFK 1), with glycogenolysis and glucose uptake being subsequently decreased due to accumulation of glucose-6-phosphate (Neely et al., 1970; Newsholme and Randle, 1964; Randle et al., 1964). Pyruvate oxidation is decreased as increased fatty acid levels inhibit the pyruvate dehydrogenase (PDH) step in the mitochondrion (Garland et al., 1962, 1964). Fatty acids inhibit the PDH complex by increasing the mitochondrial ratios of products/substrates of the reaction. This reduces the proportion of the complex in the active dephosphorylated

form by stimulating PDH kinase, and reduces the activity of this active PDH by feedback inhibition (see Randle et al., 1984, for review). In diabetes, the PDH complex is inhibited further by enhanced intrinsic PDH kinase activity and increased activity of an as yet unidentified "kinase/activator" factor which produces a more stable activation of PDH kinase (Kerbey et al., 1984, 1985; Randle et al., 1984).

The key role of fatty acid metabolism in decreasing glucose oxidation rates in diabetes has led to the development of a range of hypoglycemic drugs which block the key regulatory step in long chain fatty acid oxidation, carnitine palomitoyl transferase I (CPT I) (Cook, 1987; Stephens et al., 1984; Tutwiler et al. 1978; Wolf et al., 1982). This enzyme catalyzes the reaction producing long chain acylcarnitine from cytosolic carnitine and long chain acyl CoA. The effectiveness of some of these agents in augmenting myocardial glucose utilization in diabetes has been assessed using the isolated perfused rat heart. Tan et al. (1984) showed that the hypoglycemic sylfonylurea tolbutamide, a CPT inhibitor (Cook, 1987), increased glucose oxidation rates three-fold in glucose-perfused, isolated working hearts from chronically streptozotocin-diabetic rats. Rosen and Reinauer (1984) also demonstrated that addition of the CPT I inhibitor 2-[5-(4-chlorophenyl)pentyl]-oxirane-2-carboxylate (POCA) inhibited lipolysis and restored insulin sensitivity of glucose oxidation in glucose-perfused hearts from acutely streptozotocin-diabetic rats. However, in the chronically diabetic rat heart, Rosen et al. (1986) was unable to increase glucose utilization after POCA addition. However, the effectiveness of such

inhibitors in increasing myocardial glucose oxidation rates has not been assessed in the presence of fatty acids. This is especially pertinent in diabetics which can have both elevated serum and myocardial levels of fatty acids (Denton and Randle, 1967; Paulson and Crass, 1982).

In this study, we employed the isolated working rat heart to assess the extent to which myocardial glucose oxidation rate is suppressed by palmitate in the chronically streptozotocin-diabetic rat. We also used Etomoxir (2-[6-(4-chlorophenoxy)hexyl] -oxirane-2-carboxylate), to determine the efficacy of CPT I inhibition in increasing glucose oxidation rate in diabetic rat heart perfused in the presence of palmitate. This hypoglycemic, hypoketonemic agent is a potent new CPT I inhibitors in the class of phenylalkyloxirane carboxylic acids, and is presently being assessed for clinical use in diabetics (Wolf et al., 1982; Eistetter and Wolf, 1986, Kruszynska and Sherratt, 1986; Bliesath et al., 1987; Reaven et al., 1988). The effect of CPT I inhibition on cardiac function was also assessed.

MATERIALS AND METHODS

Etomoxir (sodium 2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylate) was a generous gift of Dr. H. Wolf, Byk Gulden Pharmazeutika, Konstanz, Federal Republic of Germany. [1-^{14}C]-palmitate (56.6 mCi.mmol^{-1}) and D-[(U)^{14}C]-glucose (1-5 mCi.mmol^{-1}) were purchased from New England Nuclear. Bovine serum albumin (fraction V) was obtained from Sigma Chemicals. All other chemicals were reagent grade.

Male Wistar rats (200-300 g) were injected via the tail vein with 65 $mg.kg^{-1}$ streptozotocin. Serum glucose was determined using Sigma kits on serum samples obtained 48 hours after streptozotocin injection and at the time of heart excision. Rats were considered diabetic if serum glucose was > 400 mg%.

Heart Perfusions:

Hearts from Na pentobarbitol anaesthetized control or diabetic rats were excised and cannulated as described previously (Lopaschuk et al., 1986; Lopaschuk and Tsang, 1987), and initially perfused retrogradely via the aorta for 10 minutes with Krebs-Henseliet buffer, pH 7.4, gassed with 95% O_2, 5% CO_2, containing 2.5 mM free calcium and 11 mM glucose. During this time the left atrium was cannulated. Hearts were then switched to the working heart mode and perfused with buffer containing either 11 mM glucose or 11 mM glucose + 1.2 mM palmitate. To measure oxidative rates, ^{14}C-glucose (600,000 $dpm.ml^{-1}$) or ^{14}C-palmitate (80,000 $dpm.ml^{-1}$) was present in the buffer. All working heart buffers contained 3% bovine serum albumin, and when used, palmitate was prebound to albumin. Perfusions were performed at a left atrial filling pressure of 15 cm H_2O and a hydrostatic aortic afterload of 80 mm Hg. Oxidative rates were determined by measuring $^{14}CO_2$ production (Lopaschuk et al., 1986). Hearts were perfused in a closed system with the gas outlet connected to a methylbenzethonium hydroxide CO_2 trap. $^{14}CO_2$ production was determined at 10 minute intervals by simultaneously collecting methylbenzethonium hydroxide samples (gaseous $^{14}CO_2$) and buffer samples via a sampling port (for ^{14}C-bicarbonate). Buffer samples were stored

under mineral oil and subsequently injected into closed metabolic flasks containing H_2SO_4. The released $^{14}CO_2$ was collected in methylbenzethonium hyroxide-filled centrewells. Methylbenzethonium hydroxide samples containing gaseous and buffer $^{14}CO_2$ were then counted on a liquid scintillation counter with quench correction. Steady-state substrate oxidation rates were obtained between 10 and 30 minutes of perfusion, and between 40 and 60 minutes in the same hearts after addition of Etomoxir at 30 minutes (final concentration of 10^{-6} M). Some hearts were perfused without radiolabelled substrate or Etomoxir for one hour to obtain control levels of tissue metabolites. Hearts were freeze-clamped at the end of perfusion with Wollenberger clamps cooled to the temperature of liquid N_2.

Tissue Analysis:

Frozen ventricular tissue was weighed and powdered in a mortar and pestle cooled to the temperature of liquid N_2. A portion of the powdered tissue was used to determine the dry to wet weight ratio. Using this ratio, as well as the total frozen ventricular weight and weight of the dried atrial tissue, total dry heart weight was calculated. Extraction of ATP, creatine-P, long

chain acyl CoA, and long chain acylcarnitine was as described previously (Lopaschuk and Spafford, 1988). Extracted long chain acyl CoA was hydrolyzed and free CoA measured fluorometrically. Extracted long chain acylcarnitine was also hydrolyzed and free carnitine measured radiometrically (McGarry and Foster, 1976). ATP, creatine-P, and citrate were determined using standard enzymatic assays (Bergmeyer and Grassl, 1983).

Statistical Analysis:

Data analysis was performed using two-way or three-way ANOVA followed by Newman-Keuls or Student's t-tests on individual groups means.

RESULTS

The effect of energy substrate on mechanical function is shown in Table 1. Heart function, as reflected by rate-pressure product, was reduced in diabetic rat hearts perfused under both substrate conditions, due to a decrease in heart rate. In diabetic rat hearts perfused in the presence of palmitate, peak systolic pressure was reduced compared to diabetic rat hearts perfused with glucose alone. Addition of Etomoxir (10^{-6} M) to either control or diabetic rat hearts perfused with glucose was without effect on the heart rate or rate pressure product. In palmitate-perfused control and diabetic rat hearts, however, Etomoxir resulted in a significant increase in heart rate and, as a result, a significant increase in rate-pressure product.

The effects of diabetes on myocardial glucose oxidation are shown in Table 2. If hearts are perfused with glucose alone, glucose

oxidation rates in diabetic rats are approximately 13-fold lower than in control rats. Addition of 1.2 mM palmitate to the perfusate of control hearts resulted in a marked drop in glucose oxidation to rates similar to those seen in diabetic rat hearts perfused in the absence of palmitate. If palmitate is added to the perfusate of diabetic rat hearts, glucose oxidation is almost completely abolished. The effect of palmitate and diabetes on glucose oxidation remains if glucose oxidation rates are corrected for differences in work performed by the hearts (Table 1).

If 10^{-6} M Etomoxir is added to the perfusate, glucose oxidation rates per gram dry weight are significantly increased by approximately two-fold under all experimental conditions except in palmitate-perfused diabetic hearts, where a 1.63 fold increase was observed. If glucose oxidation rates were corrected for cardiac work, the stimulation of glucose oxidation by Etomoxir remained in all experimental groups except in diabetic, palmitate-perfused, where no significant change was observed.

Oxidation rates of exogenous palmitate in control and diabetic rat hearts are shown in Table 3. No difference in palmitate oxidation was observed between control and diabetic rat hearts, whether expressed on per gram dry heart weight or per unit cardiac function. This supports earlier work in which we demonstrated no difference in myocardial palmitate oxidation rates between normal and genetically diabetic "BB"/Wistar rats (Lopaschuk and Tsang, 1987). Etomoxir did not inhibit exogenous palmitate oxidation in either controls or diabetics. In fact, even when rates were corrected for increased cardiac function, a significant increase in palmitate

oxidation occurred in diabetic rat hearts after Etomoxir.

The relative contribution of exogenous glucose and palmitate oxidation to ATP generation in control and diabetic rat hearts is presented in Table 4. ATP generation was calculated using the values of 38 ATP per molecule of glucose oxidized and 129 ATP per molecule of palmitate oxidized. In diabetic rat hearts perfused with glucose alone, ATP production from glucose is markedly depressed, suggesting that the majority of ATP production under these conditions is derived from endogenous triacylglycerols. In both control and diabetic rat hearts perfused with glucose alone, Etomoxir doubled ATP production from exogenous glucose. In control hearts perfused in the presence of 1.2 mM palmitate, ATP generated from exogenous glucose oxidation is only 6% of that generated from exogenous palmitate oxidation. In diabetic rat hearts perfused under the same conditions, ATP from exogenous glucose oxidation represents a mere 0.6% of that from oxidation of perfusate palmitate. The balance of energy provision is shifted only slightly under these conditions by Etomoxir, the percent ATP provided by exogenous glucose/palmitate increasing about 1.6 times in controls, and remaining essentially the same in diabetics.

Tissue levels of intermediates of the CPT I reaction measured in hearts freeze-clamped after perfusion under the various experimental conditions, are shown in Figures 1 and 2. As expected, addition of palmitate to the perfusate increased levels of long chain acyl CoA and long chain acylcarnitine in control hearts. Levels of long chain acyl CoA were elevated higher in diabetic versus control glucose-perfused rat hearts, and were surprisingly reduced in diabetics by the presence of palmitate. Long chain acylcarnitine

levels were not elevated in diabetic rats hearts perfused with glucose and were not increased in these hearts if palmitate was added to the perfusate. Etomoxir reduced levels of long chain acyl CoA in control rat hearts perfused in the presence of palmitate, and diabetic rat hearts perfused in the absence of palmitate. Etomoxir did not reduce levels of long chain acylcarnitine under any experimental condition.

Accumulation of citrate during oxidation of lipid fuels is thought to suppress glucose utilization by inhibiting PFK 1. Figure 3 shows citrate levels from tissue obtained from hearts frozen following perfusion. As expected, palmitate increases the concentration of citrate in both control and diabetic rat hearts. Citrate levels were not increased in diabetic rat hearts under either substrate condition. Etomoxir was without effect on citrate levels except in control rat hearts perfused with glucose, where a significant increase was observed.

DISCUSSION

This study demonstrates the important role of fatty acids in regulating myocardial glucose oxidation in normal and diabetic rats. Addition of free fatty acids (1.2 mM palmitate) to isolated working hearts from non-diabetic rats perfused with glucose resulted in a marked reduction (13-fold) in glucose oxidation rates, to levels seen in chronically diabetic rat hearts perfused in the absence of added fatty acids. We also demonstrate that the CPT I inhibitor, Etomoxir, can increase myocardial glucose oxidation, with a concomitant increase in cardiac funtion in both control and diabetic, fatty

acid-perfused rat hearts. The data demonstrating the effect of fatty acids on glucose oxidation support the work of Randle and coworkers (Newsholme and Randle, 1964; Randle et al., 1964; Garland et al., 1962, 1964; Garland and Randle, 1964) who demonstrated that addition of fatty acids or ketone bodies to Langendorff perfused rat hearts reduces glucose utilization at the sites of glucose uptake, glycolysis, and pyruvate oxidation. We have extended these studies to the isolated working heart in which cardiac function can be measured. We also demonstrate that in diabetic rat hearts, glucose oxidation is almost completely suppressed by a physiologically obtainable concentrations of exogenous free fatty acids.

The low glucose oxidation rates in glucose-perfused diabetic rat hearts compared to control hearts perfused under the same conditions may be explained by an increased reliance on fatty acid oxidation. In acutely diabetic rat hearts, inhibition of glucose metabolism can be almost entirely explained by short-term, reversible effects of increased lipid oxidation. Our results parallel those of Randle's group, in which glucose oxidation rates seen in diabetic rat hearts can be produced by perfusion of normal rat hearts with fatty acids (Newsholme and Randle, 1964; Randle et al., 1964; Garland et al., 1962, 1964; Garland and Randle, 1964). The decrease in glucose oxidation in diabetic rat hearts perfused in the absence of added palmitate may be explained by an increased metabolism of fatty acids from endogenous triacylglycerols (Denton and Randle, 1967). Indeed, tissue levels of fatty acids stored in the form of triacylglycerols are markedly elevated in the diabetic rat heart. This occurs in part due to higher circulating fatty acid concentrations, which both

stimulates synthesis of, and inhibit hydrolysis of triacylglycerols (Denton and Randle, 1967; Paulson and Crass, 1982). In addition, myocardial total CoA levels are elevated in diabetes (Reibel et al., 1981), which favours storage of long chain fatty acids as triacylglycerols instead of transport across the mitochondrial membrane for oxidation (Lopaschuk et al., 1986; Lopaschuk and Tsang, 1987).

Addition of palmitate to the perfusate of diabetic rat hearts resulted in an almost complete suppression of glucose oxidation (Table 2). This decrease cannot be accounted for by an increase in oxidation of either exogenous long chain fatty acids (Tables 3 and 4) or short chain fatty acids (Barbee etal., 1988) in diabetic rat hearts. In the presence of palmitate, endogenous fatty acid oxidation from stored triacylglycerols has not been shown to be elevated in diabetic rat hearts (Paulson and Crass, 1982). In addition, analysis of whole tissue levels of long chain acyl CoA, long chain acylcarnitine, and citrate suggest that flux of fatty acids through the carnitine acyltransferases and the TCA cycle was no greater in these chronic diabetics and that sensitivity of CPT I to inhibition by Etomoxir was no different in controls and diabetics. The inability of diabetic rat hearts to oxidize glucose without a concomittant increase in fatty acid oxidation may result in a decreased ability of the heart to maintain metabolic demand under certain conditions. Perfusion with fatty acids decreased peak systolic pressure and tended to decrease HRXPSP in diabetic rat hearts. We have observed that chronically diabetic hearts perfused in the presence of fatty acids could not maintain PSP at the

same rate of electrical pacing as in the absence of fatty acids (Lopaschuk and Spafford, unpublished). This may be related to a limitation of energy production by suppressed glucose oxidation. A direct study of the effects of fatty acids on the ability of chronically diabetic rat hearts to perform at maximal workloads has not been carried out, however.

The inhibition of myocardial glucose oxidation in diabetes may occur by a combination of several mechanisms. Besides an inhibition of glucose uptake, citrate accumulation as a result of fatty acid oxidation may be critical in the inhibition of glycolysis at the phosphofructokinase step, resulting in further depression of glucose uptake and glycogenolysis (Randle et al., 1963; Neely et al., 1970). However, there is evidence to suggest that in the long-term diabetic model a chronic adaptation in myocardial lipid handling mechanisms may occur. For instance, Rosen et al. (1986) showed that in contrast to elevated rates of endogenous lipid oxidation and increased citrate levels in glucose-perfused hearts from acutely diabetic rats, values were no different from controls in chronically diabetic rat hearts. We demonstrate here that citrate levels are not elevated in the chronically diabetic model (Figure 4). Another mechanism for the decreased glucose oxidation in diabetes is the lower %PDHa, mediated by oxidation of fatty acids, which increases acetyl CoA/CoA ratio during fatty acid oxidation. Caterson et al. (1982) demonstrated that normalization of %PDHa in glucose-perfused acutely diabetic rat hearts by insulin and the CPT I inhibitor TDGA is accompanied by a normalization of the ratio of acetyl CoA/CoA. We did not measure acetyl CoA levels in our hearts, but suggest that,

similar to citrate, acetyl CoA levels may not necessarily be increased in the chronically diabetic rat heart. The data of Caterson et al. (1982) also do not rule out that %PDHa could be reduced by factors in diabetes other than acute alterations in acetyl CoA/CoA, since it was not stated whether acetyl CoA/CoA was normalized by TDGA in diabetic hearts perfused without glucose alone, where %PDHa was not normalized. Miller et al. (1987) demonstrated that TDGA acutely increases acetyl CoA in fatty acid-perfused swine hearts.

In this study we determined the effect of CPT I inhibition on myocardial glucose oxidation rates in both control and diabetic rats. Addition of Etomoxir increased myocardial glucose oxidation rates in all experimental conditions. Our results contrast with those of Rosen et al. (1986) who failed to demonstrate any increase in glucose oxidation in chronically diabetic rat heart, using the CPT I inhibitor, POCA. However, Rosen's group found little difference in glucose oxidation between controls and diabetics, an observation which contrasts the results of our study and of other groups (Randle et al., 1964; Tan et al., 1984). Although Etomoxir did not completely inhibit the acute effects of palmitate on glucose oxidation in control hearts, it did improve mechanical function. Heart rate and rate-pressure product were increased by Etomoxir in palmitate-perfused hearts from control and diabetic rats. This supports the abovementioned concept that fatty acids may have depressive effects on cardiac function which are acutely reversible and mediated by effects on glucose utilization.

This study emphasizes the importance of fatty acids in

regulating myocardial glucose oxidation, especially in diabetes. Glucose oxidation is substantially reduced in normal rat hearts by the presence of fatty acids. Glucose oxidation is almost completely suppressed in diabetic rat hearts perfused with the same fatty acid concentration, which is in the range of serum free fatty acids observed in diabetes. CPT I inhibition resulted in an increase in glucose oxidation rates and a slight increase in cardiac function in hearts perfused with fatty acids. These data suggest that acutely reversible effects of fatty acids can contribute to decreased myocardial glucose oxidation and cardiac function in diabetes.

Table 1: Effect of palmitate and Etomoxir on heart function in isolated working hearts from control and diabetic rats.

Perfusion Condition	Heart Rate ($beat.min^{-1}$)	Peak Systolic Pressure (mm Hg)	Heart Rate X Peak Systolic Pressure ($X10^{-3}$)
CONTROL			
1) 11 mM glucose	204.9±10.8	114.9±2.5	23.5±1.1
+ Etomoxir	217.1±6.2	110.8±1.8a	24.0±0.7
2)11 mM glucose, 1.2 mM palmitate	201.1±9.2	111.3±2.1a	22.3±0.8
+ Etomoxir	225.8±6.9a	106.5±1.7	24.1±0.8a
DIABETIC			
1) 11 mM glucose	140.0±6.7*	126.4±3.9	17.6±0.8*
+ Etomoxir	160.0±15.8	119.0±7.8	18.7±0.6
2) 11 mM glucose, 1.2 mM palmitate	128.0±13.1*	111.6±3.7t	14.2±1.4*
+ Etomoxir	155.0±10.5a	105.7±1.9	16.3±1.1a

Heart rate, peak systolic pressure, and the heasrt rate-peak systolic pressure product were determined in hearts perfused at a 15 cm H_2O preload and 80 mm Hg afterload. Etomoxir, when used, was added to the perfusate at concentration of 10^{-6} M. *, $p<0.05$ diabetic versus control; t, $p<0.05$ palmitate versus glucose; a, $p<0.05$ Etomoxir versus paired group without Etomoxir.

Table 2: Effect of palmitate and Etomoxir on glucose oxidation rates in isolated working hearts from control and diabetic rats.

	CONTROL	DIABETIC
Perfusion Condition	$^{14}CO_2$ production (nmol glucose. g dry^{-1}.min^{-1})	$^{14}CO_2$ production (nmol glucose. g dry^{-1}.min^{-1}
1) 11 mM glucose	1127±140	89±11*
+ Etomoxir	2146±214a	195±41a
2) 11 mM glucose, 1.2 mM palmitate	89±19t	9±2*t
+ Etomoxir	192±38a	15±3a
	$^{14}CO_2$ production (nmol glucose.min^{-1}. (HRXPSPX10^{-3})$^{-1}$)	$^{14}CO_2$ production (nmol glucose.min^{-1}. (HRXPSPX10^{-3})$^{-1}$)
1) 11 mM glucose	14.57±1.88	1.19±0.22*
+ M Etomoxir	26.83±2.66a	2.50±0.52a
2) 11 mM glucose, 1.2 mM palmitate	1.23±0.15t	0.24±0.07*t
+ Etomoxir	2.52±0.50a	0.31±0.08

Hearts are the same as those described in Table 1. Glucose oxidation was measured as described in Methods and expressed per gram dry heart weight, or corrected for work performed by the heart. *, $p<0.05$ diabetic versus control; t, $p<0.05$ palmitate versus glucose; a, $p<0.05$ Etomoxir versus paired group without Etomoxir.

Table 3: Effect of Etomoxir on palmitate oxidation rates in isolated working hearts from control and diabetic rats.

Perfusion Condition	CONTROL	DIABETIC
	$^{14}CO_2$ production (umol palmitate.g dry^{-1}.min^{-1})	
11 mM glucose, 1.2 mM palmitate	453±51	403±49
+ Etomoxir	601±61a	593±68a
	$^{14}CO_2$ production (nmol palmitate.min^{-1}.(HRXPSPX10^{-3})$^{-1}$)	
11 mM glucose, 1.2 mM palmitate	7.10±0.89	5.66±0.43
+ Etomoxir	8.88±1.25	7.22±0.49a

Hearts are the same as those described in Table 1. Palmitate oxidation was measured as described in Methods and expressed per gram dry heart weight, or corrected for work performed by the heart. *, $p<0.05$ diabetic versus control; t, $p<0.05$ palmitate versus glucose; a, $p<0.05$ Etomoxir versus paired group without Etomoxir.

Table 4: Calculated ATP produced from oxidation of exogenous glucose and palmitate in isolated working hearts from control and diabetic rats.

ATP Production (nmol. g dry^{-1}.min^{-1})

	CONTROL		DIABETIC	
Perfusion Condition	From Glucose	From Palmitate	From Glucose	From Palmitate
1) 11 mM glucose	42830		3372	
+ Etomoxir	82547		7399	
2) 11 mM glucose, 1.2 mM palmitate	3379	58476(5.8)	349	52039(0.67)
+ Etomoxir	7309	77581(9.4)	568	76484(0.74)

Data are derived from the same hearts as described in Table 1. ATP production was calculated from theoretical ATP yeilds of 38 and 129 for complete oxidation of glucose and palmitate, respectively, as described in Methods. Values in parentheses are the ratio of ATP produced from exogenous glucose/palmitate for each condition.

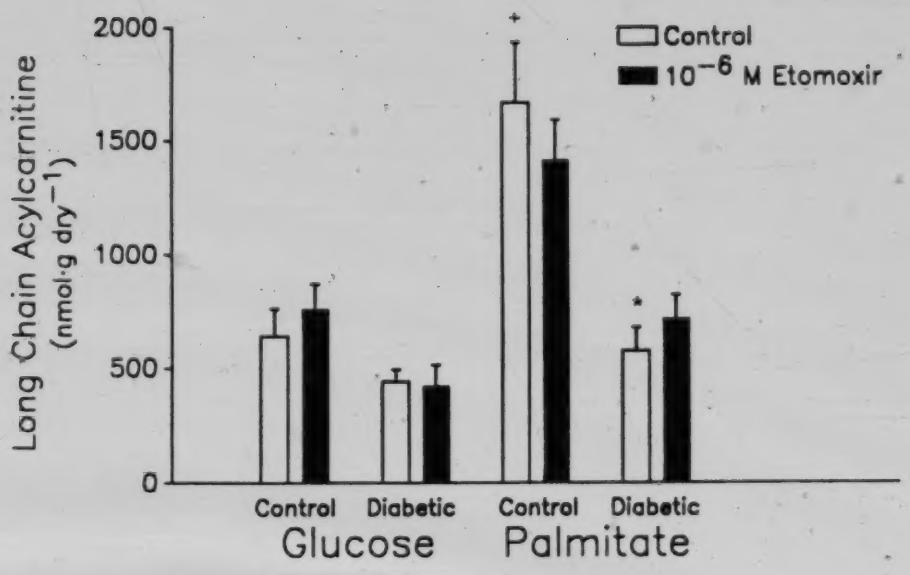

Figure 1: Effect of Etomoxir and palmitate on long chain acylcarnitine levels in isolated working hearts from control and diabetic rats.

Values are obtained from hearts freeze-clamped after perfusion. Hearts were perfused with 11 mM glucose ± 1.2 mM palmitate either for a 30 minute control period without Etomoxir, followed by a 30 minute period after addition of Etomoxir (as described in Table 1), or 1 hour without Etomoxir (see Materials and Methods). Values are mean ± SEM of at least 5 hearts. Glucose= 11 mM glucose; Palmitate= 11 mM glucose + 1.2 mM palmitate. *, $p<0.05$ diabetic versus control; t, $p<0.05$ palmitate versus glucose; a, $p<0.05$ Etomoxir versus unpaired control without Etomoxir.

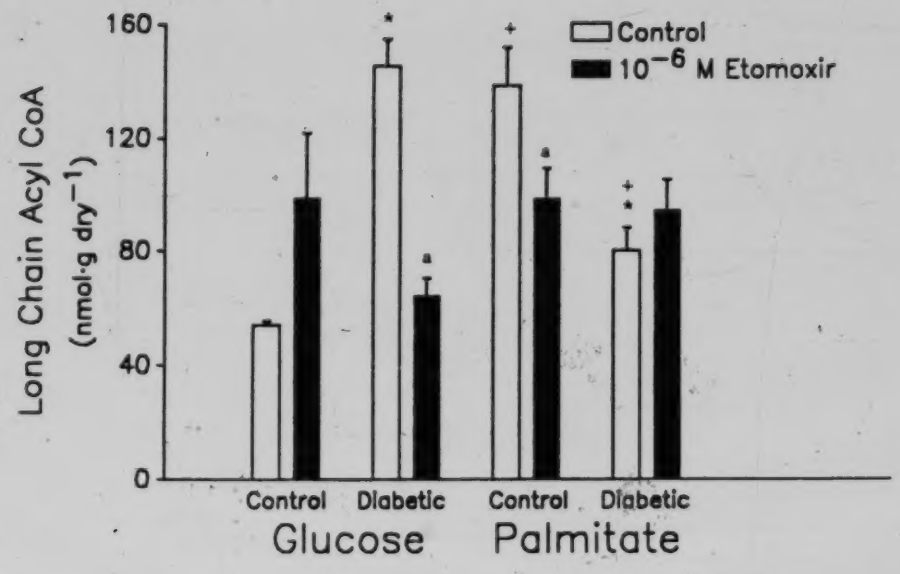

Figure 2: Effect of Etomoxir and palmitate on long chain acyl CoA levels in isolated working hearts from control and diabetic rats.

Hearts are the same as those described in Figure 1. *, $p<0.05$ diabetic versus control; t, $p<0.05$ palmitate versus glucose; a, $p<0.05$ Etomoxir versus unpaired control without Etomoxir.

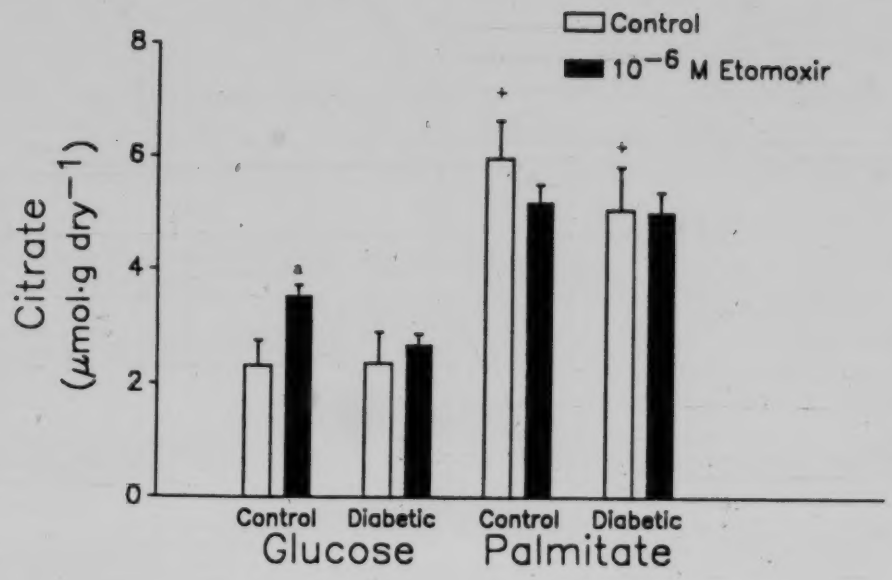

Figure 3: Effect of Etomoxir and palmitate on citrate levels in isolated working hearts from control and diabetic rats.

Hearts are the same as those described in Figure 1. *, $p<0.05$ diabetic versus control; t, $p<0.05$ palmitate versus control; a, $p<0.05$ Etomoxir versus unpaired control without Etomoxir.

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CHAPTER 4: GENERAL DISCUSSION

Long-Term Regulation of Glucose Oxidation and Function in Diabetic Rat Heart:

The acute and chronic factors responsible for decreased cardiac performance in diabetic animals remain to be fully elucidated. Reduced contractility in the chronically diabetic rat heart is largely due to changes in myosin isozyme predominance, from the normally high ATPase V1 to the lower ATPase V3 isozyme (Dillmann, 1980). Signals from reduced thyroid hormone levels and altered cardiac metabolism contribute to this shift in myosin isozyme expression in diabetes. The role of triiodothyronine (T3) in regulating myosin isozyme phenotype is fairly well characterized. A distinct metabolic signal, probably generated by carbohydrate metabolism, also regulates myosin isozyme expression. It was hypothesized that chronic administration of an agent which directly activates the PDH complex to diabetic rats would result in increased glucose oxidation and subsequently increased myofibril ATPase activity. DCA increases the percentage of the PDH complex in the active form (%PDHa) by inhibiting PDH kinase (Whitehouse et al., 1974). In the first study DCA was administered chronically in an attempt to provide a long-term reactivation of the PDH complex in diabetic rat hearts. This study demonstrates that long-term administration of high doses of DCA can increase glucose oxidation in

isolated working hearts from diabetic rats, even in the presence of fatty acids. Depressed cardiac Ca^{2+}-activated myofibril ATPase seen in the untreated diabetic rats was completely normalized by DCA treatment. This effect was not due to increased serum T4 levels in DCA-treated diabetic rats. These data suggest that a metabolic signal generated by increased flux through PDH induces altered activity of contractile proteins. Diabetes and DCA did not alter the calcium sensitivity (pCa_{50}) of myofibril ATPase, but rather the maximal Ca^{2+}-activated, Mg^{2+}-stimulated myofibril ATPase. This can be explained mainly by effects on basal Mg^{2+}-stimulated myofibril ATPase, although a clear interpretation of the mechanism of altered maximal myofibril ATPase activity is not apparent from the data. As suggested by Belcastro et al. (1985), an adaptation in response of the myofibril ATPase to Mg^{2+} may therefore have taken place in diabetes, which was also ameliorated by DCA treatment. Giacomelli and Weiner (1979) have noted alterations in the thin filament of the myofibril in the diabetic rat heart, which may relate to altered regulation of myofibril ATPase. It is likely that a change in myosin isozyme predominance from V1 to V3 can account for most of the decrease in myofibril ATPase in diabetics (Dillmann, 1980; Malhotra et al., 1981), although this was not measured in the present study. An increase in V1 myosin and a decrease in V3 myosin content may also account for increased myofibril ATPase in DCA-treated diabetics. Methylpalmoxirate treatment can also increase %V1 myosin and Ca^{2+}-myosin ATPase in diabetic rat hearts (Dillmann, 1985). It was speculated that this effect was due to an increase in glucose oxidation as a result of inhibition of fatty acid oxidation.

Another factor that must be considered is altered regulation of V1 myosin itself. Winegrad and Weisberg (1987) have recently demonstrated that V1 myosin ATPase activity is increased by cyclic AMP, while V3 myosin is unaffected.

While cardiac myofibril ATPase activity was decreased in diabetic rats and normalized by DCA-treatment, corresponding changes in rate-pressure product were not observed in the sames hearts perfused as isolated working hearts. This would suggest that myofibril ATPase activity is not always directly correlated with contractile function. This dissociation between contractile protein activity and function has also been shown in hearts from diabetic rats fed methylpalmoxirate, where V1 myosin was increased but isolated papillary muscle contractility was not similarly normalized (Popovich et al., 1987). Similarly, Tahiliani and McNeill (1985) were unable to demonstrate improved cardiac function (as assessed by left ventricular developed pressure in response to increasing left atrial filling pressure) in diabetic rats fed methylpalmoxirate. In this last study, depressed sarcoplasmic reticular calcium transport was prevented by the drug treatment. Function in the isolated working heart is also determined by factors other than the contractility, as measured directly by mechanical Vmax of isolated cardiac muscle. Vmax may be closely related to isomyosin pattern and myofibril ATPase activity. However, changes in the time course of development of peak tension or shortening velocity (measured in isolated muscle preparations) or in intrinsic heart rate (i.e., in the isolated working heart) may be due to factors other than myofibril or sarcoplasmic reticulum function. Prolongation of time

to peak shortening was shown to be correlated with increased action potention duration in hearts undergoing hypertrophy from renal hypertension (Capasso et al., 1982). Abnormalities in action potential development and response to calcium have been observed in diabetic rat hearts (Sauviat and Feuvray, 1986). These observations may be related to consistently reduced heart rate in diabetic rats, which can occur within 48 hours of induction of streptozotocin diabetes, or within 24 hours after insulin withdrawal of insulin in spontaneously diabetic rats (Lopaschuk and Spafford, 1988; Lopaschuk and Tsang, 1987). Heart rate was also reduced in diabetic rats in the present study, and remained unaltered by DCA treatment. Similarly, many of the abovementioned biochemical derangements in the myocardium of diabetic rats may been unaffected DCA-treatment. The presence of high palmitate levels in the perfusion buffer may decrease rate-pressure product in isolated working hearts from diabetic as well as control animals (Wall and Lopaschuk, 1988; Fields et al., 1986). Therefore, differences in rate-pressure product between control and diabetic rat hearts may have been masked by this condition in the present study. The lack of increase in cardiac performance in DCA-treated diabetic rats may be due to a protein catabolic state in caused by the combined effects of low food intake and DCA. Fields et al. (1987) demonstrated that loss of lean body mass and nitrogen in starving rats was accelerated by DCA administration, which was suggested to increase gluconeogenesis from amino acids as a result of its glucose-wasting effect. Thus, cardiac performance was reduced in both control and diabetic rats treated with DCA, possibly due to reduced heart size and myofibril protein

yield.

Glucose oxidation rates measured in isolated working hearts in the presence of 1.2 mM palmitate and no insulin were depressed in diabetic versus control rat hearts. This was partially reversed by chronic DCA-treatment. Therefore, it can be reasonably inferred that depressed myocardial glucose oxidation rates in diabetics would be increased to at least the same degree in vivo by the DCA-treatment regime. It is also plausible to assume that %PDHa was increased by the DCA treatment, since this agent has a specific action on PDH (Whitehouse et al., 1974). Suppression of this step is of key importance in diminishing myocardial glucose oxidation in diabetes (Randle et al., 1984). It is unknown whether the effects of DCA in vivo were diminished upon excision of hearts and perfusion in the absence of DCA. Glucose oxidation rates did not decline over the 30 minute perfusion period in hearts from DCA-fed diabetic rats. However, Whitehouse et al. (1974) showed that in normal hearts perfused with 1 mM DCA in addition to glucose, acetate, and insulin, switching to DCA-free medium after 6 minutes resulted in reversal of DCA effects on %PDHa after about 5 minutes. Nevertheless, if the effects of circulating DCA were reduced by perfusion without DCA, the observed increase in glucose oxidation in DCA-fed diabetics could only be an underestimate of the increase in glucose oxidation due to DCA in vivo. When 0.5 mM DCA was added to the perfusate, glucose oxidation rates were increased maximally in palmitate-perfused hearts from diabetic rats, reaching rates seen in control hearts in the absence of palmitate. This demonstrates the key involvement of the PDH complex in depressed glucose oxidation in diabetic rat heart.

The nature of the metabolic signal that alters myocardial protein expression in the diabetic cannot be clearly deduced from studies in which chronic drug administration to alter substrate fluxes is carried out. DCA does activate the PDH complex, thereby increasing flux of pyruvate to the TCA cycle for oxidation. However, it also has secondary effects on other metabolic pathways. DCA decreases fatty acid utilization by the in situ canine heart, and reduces the inhibitory effect of fatty acids on extraction of glucose, pyruvate, and lactate (McAllister et al., 1973; Mjos et al., 1976). DCA also decreases $^{14}CO_2$ production and diminishes specific activity of acetyl CoA during perfusion with glucose and ^{14}C-acetate (McAllister et al., 1973). The redox state of the cytosol can be affected since DCA increases the lactate/pyruvate ratio (Higgins et al., 1978). Glycolytic rates can also be increased, especially during fatty acid oxidation, occuring in part due to decreased citrate levels (McAllister et al., 1973). As with the CPT I inhibitor, methylpalmoxirate, which reverses the myosin isozyme switch in diabetes (Dillmann, 1985), the reversal of depressed myofibril ATPase in diabetes by DCA could be related to an inhibition of fatty acid oxidation or an increase in glycolytic rate, as opposed to increasing pyruvate oxidation by reactivating the PDH complex.

It is interesting that a specific group of glucose regulated proteins are induced by glucose starvation in a variety of cells (see Lee, 1987 for review). These proteins are produced under a variety of stress conditions such as calcium ionophore administration and anaerobiosis. They are related to the heat shock/stress proteins,

which can be induced in cardiac tissue by ischemia (Dillmann et al., 1986; Curry, 1987) and conditions which elevate tissue lactate concentrations, such as aortic stenosis (Hammond et al., 1982). In diabetes, the redox state of the cytosol is also altered. The connection between heat shock/stress proteins and glucose regulated proteins remains elusive. However, it is noteworthy that in both diabetes and in response to aortic stenosis, the isozyme shift from V1 to V3 myosin occurs (Dillmann, 1980; Swynghedauw et al., 1980; Umeda et al., 1987; Izumo et al., 1987). V3 myosin has higher mechanical efficiency, in terms of ATP hydrolyzed per unit of force developed (Holubarsch et al., 1985). Perhaps the production of these proteins is related to the signalling of a metabolic compromise, which induces proteins such as the V3 myosin, which allow conservation of cellular energy. Mariash and Oppenheimer (1985) have shown that DCA can reproduce the effects of glucose, which induces malic enzyme in hepatocytes. This suggests that the signal inducing this carbohydrate-regulated gene is generated at the PDH complex. It stands to reason that such a highly regulated step in cellular metabolism might signal the energy status of the cell, since the fate of glucose is irreversibly determined at PDH (Randle et al., 1978; Randle, 1986).

Although the cardiac myosin heavy chains are regulated by T3, and probably a metabolic signal, the site at which this interaction occurs is open for speculation. The phosphoenolpyruvate carboxykinase gene is regulated by interaction of both insulin and T3 with 5' regulatory sequences of the gene (Wynshaw-Boris et al., 1986). Diabetes results in reduced T3 receptor levels in liver

(Murty et al., 1986), which could reduce the T3 effect in the nucleus. As discussed in Chapter 2, similar to diabetes, a signal related to energy metabolism may cause the decreased V1 and increased V3 myosin content in hearts undergoing cardiac hypertrophy due to aortic stenosis. However, is seems unlikely that the metabolic signal to isomyosin switches would act by reducing T3 receptor levels during aortic stenosis.

Acute Effects of Fatty Acids on Myocardial Glucose Oxidation and Cardiac Function in Diabetes:

The relative contribution of acute and chronic effects of increased reliance on fatty acid oxidation to decreased myocardial glucose oxidation and cardiac function in diabetes is not understood. A key regulatory step in this inhibition is the PDH complex, whose activity is reduced via phosphorylation by increased PDH kinase activity (Kerbey et al., 1984; Randle et al., 1984). While fatty acid metabolism can acutely increase PDH kinase activity and decrease %PDHa, mainly by increasing acetyl CoA/CoA, the factors responsible for the additional increase in PDH kinase and "kinase/activator" factor in diabetes are unknown. In cultured hepatocytes, octanoate and glucagon at concentrations which approximates starvation or diabetes can increase PDH kinase activity to levels seen under these conditions in vivo (Fatania et al., 1986). The effects of long-term inhibition of fatty acid oxidation by blocking entry of long chain fatty acids into the mitochondrion at CPT I, on myocardial glucose oxidation have not been determined. In fact, prior to the present study, the effects of acute CPT I inhibition on glucose oxidation in the isolated heart had not been

characterized in the presence of competing fatty acid substrates which would be present in vivo. In addition, fatty acids may have acute effects on heart function at high workloads in diabetes. Therefore, in the second part of this thesis, the effects of palmitate and of the CPT I inhibitor, Etomoxir, on glucose oxidation rates and cardiac function were determined in isolated working hearts from normal and chronically diabetic rats.

Glucose oxidation rates were highly dependent on the presence of palmitate and on the diabetic condition. In control rat hearts, the presence of palmitate reduced glucose oxidation to levels seen in diabetic rat hearts perfused without palmitate, while perfusion with palmitate virtually eliminated glucose oxidation in diabetic rat hearts. The lower glucose oxidation rates in diabetics in the glucose-perfused condition may have been due to greater endogenous fatty acid oxidation rates from increased myocardial triacylglycerol stores (Denton and Randle, 1967; Lopaschuk and Tsang, 1987), with the resultant production of intermediates which are inhibitory to glucose oxidation. Levels of long chain acyl CoA, long chain acyl carnitine, and citrate were not higher in diabetic rat hearts perfused with glucose. However, whole tissue concentrations of intermediates of fatty acid metabolism do not necessarily reflect rates of fatty acid oxidation. Oxidation rates of endogenous fatty acids from stored triacylglycerols in a glucose-perfused heart can be calculated by measuring myocardial oxygen consumption, and calculating glycolytic rates and glucose oxidation from exogenous glucose and stored glycogen, by measuring metabolites of these pathways. The remainder of oxygen consumption unaccounted for is attributed to endogenous

fatty acid oxidation (Denton and Randle, 1967). Since this is not a direct measurement, reduced glucose oxidation rates in diabetic rat hearts perfused with glucose cannot be definitely attributed to higher fatty acid oxidation rates. In support of this, levels of citrate, which can inhibit glycolysis, were not higher in diabetic rat hearts in the present study.

Perfusion with 1.2 mM palmitate drastically decreased glucose oxidation rates. Levels of intermediates of fatty acid oxidation, long chain acyl CoA, long chain acylcarnitine, and citrate, were increased in palmitate-perfused hearts, supporting the concept that glucose oxidation was reduced due to increased fatty acid oxidation. The almost complete suppression of glucose oxidation in palmitate-perfused diabetic rat hearts may have been a result of increased fatty acid oxidation rates from combined endogenous and exogenous sources. This study, is the first to measure exogenous palmitate oxidation rates directly in experimental diabetes. Palmitate oxidation, measured as $^{14}CO_2$ production from ^{14}C-palmitate in the perfusate, was no different in control and diabetic rat hearts, taking into account the lower work output of the diabetic rat hearts. This corresponds with previous work by Lopaschuk and Tsang (1987), who observed no difference in palmitate oxidation rates between control and spontaneously diabetic "BB" Wistar rats. It is unresolved whether endogenous fatty acid oxidation is increased in diabetic rat hearts under these conditions. In the presence of fatty acids, levels of long chain acyl CoA, long chain acylcarnitine, and citrate were also not higher in diabetics versus controls. Endogenous fatty acid oxidation can be

assessed more directly by perfusing with ^{14}C-labelled fatty acid to prelabel the triacylglycerol pool, and then perfusing with unlabelled substrate(s), subsequently collecting $^{14}CO_2$ to measure oxidative rates. Using this method, Paulson and Crass (1982) observed that endogenous fatty acid oxidation rates were no different in control rat hearts perfused with a low fatty acid concentration and diabetic rat hearts perfused with a high fatty acid concentration. It is unresolved whether endogenous fatty acid oxidation rates would have been lower in control hearts if they had been perfused with the higher fatty acid concentration, which would inhibit triacylglycerol lipolysis. In this type of experiment, relative oxidation rates must be related to the work or metabolic rate of the heart, which is lower in diabetic rat hearts. Thus, it cannot be concluded from this study whether glucose oxidation rates in the presence of palmitate are lower in diabetic versus control rat hearts due to increased fatty acid oxidation.

It is well-established that cardiac function is impaired in diabetic rat hearts (Ingebretsen et al., 1980; Vadlamudi et al., 1982; Penparkgul et al., 1981; Garber and Neely, 1983; Lopaschuk and Tsang, 1987). This can be attributed to reduced left ventricular developed pressure and positive and negative dP/dt, as well as decreased intrinsic heart rate in the isolated working heart. This is the first study to assess cardiac function in diabetic rat hearts in the presence of high fatty acid levels. In diabetic rat hearts, peak systolic pressure was decreased in the presence of palmitate. In diabetic rat hearts there was as trend towards a lower performance (HRXPSP) in the presence of palmitate, which was accompanied by

significantly reduced heart rate. A critical rate of glucose utilization may be necessary for optimal functioning of membrane ion transport or other systems related to intrinsic rate of the heart or contractility, as suggested by Opie and Bricknell (1979). Glucose utilization would be reduced by high fatty acid levels and in diabetes. If a suppressive effect of fatty acids on function is mediated by reduced glucose oxidation, then it follows that fatty acids would have a more deleterious effect in the diabetic rat heart, where glucose oxidation is almost completely shut down by fatty acids. Energy from oxidation of fatty acids is required for performance of high levels of cardiac work (Pearce et al., 1979; Neely and Morgan, 1974). However, high rates of fatty acid utilization at the expense of glucose utilization may suppress cardiac function. A recent study by Lopaschuk and Spafford (1988) showed that pressure development could not be maintained in palmitate-perfused chronically diabetic rat hearts at the same rate of pacing as in the absence of palmitate. The requirement for increased glucose oxidation at high rates of cardiac work may not be met in hearts using fatty acids for a high proportion of energy provision, as in the case of diabetes. The present study shows that glucose oxidation rates are practically non-existent under the same preload and afterload and substrate conditions, but without pacing. Etomoxir increased cardiac function in palmitate-perfused control and diabetic hearts, primarily due to an increase in heart rate. Etomoxir also increased glucose oxidation rates. Miller et al. (1987) observed in in situ aerobically perfused heart from normal swine, that the deleterious effects of high serum fatty acid levels

on regional work and systolic and total shortening could be prevented by the CPT I inhibitor, tetradecylglycidic acid. They assumed that glucose oxidation increased in hearts treated with tetradecylclycidic acid, since palmitate oxidation decreased and oxygen consumption remained unchanged. They concluded that this may have exerted a beneficial effect on aerobic heart function. Other studies have also shown increased cardiac function after CPT I inhibition (Tan et al., 1984; Rosen et al., 1986a). However, some researchers have either shown no effect or a detrimental effect of CPT I inhibition on the ability of the normal heart to perform high workloads (Pearce et al., 1979; Bielefeld et al., 1985; Rosen and Reinauer, 1984). The effect of CPT I inhibition on cardiac function appears to be dependent on the levels of competing fatty acid substrate, and the work demand. For example, if high fatty acid levels are present and glucose oxidation is impaired, then an appropriate concentration of CPT I inhibitor would allow an increase in glucose oxidation without inhibiting fatty acid oxidation to an extent that would impair work capacity, as suggested by the present study. On the contrary, if fatty acid levels are not increased and glucose oxidation is normal, then the same concentration of CPT I inhibitor might block fatty acid oxidation to an extent that would impair energy delivery and limit cardiac function at high workloads, as suggested by Pearce et al. (1979). It must be considered also that the inhibitory effect of these agents depends on the concentration of competing fatty acid substrate.

The mechanism by which Etomoxir increases glucose oxidation did not involve a reduction in levels of citrate. Exogenous fatty

acid oxidation does not appear to be inhibited by Etomoxir at this concentration. This is demonstrated by direct measurement of ^{14}C-palmitate oxidation rates. However, endogenous fatty acid oxidation rates may have been inhibited by Etomoxir, especially in glucose-perfused hearts, where competition from exogenous fatty acids in the perfusate was not present. Etomoxir also increased the overall rate of metabolism. It appears that in both fatty acid and glucose oxidation increased to meet the increased energy demand, since oxidative rates of both substrates increased on an absolute (per gram dry weight) basis. However, glucose oxidation was still increased when corrected for the increase in work after Etomoxir, suggesting that its relative contribution to energy supply was increased by Etomoxir. Increased TCA cycle activity with increased metabolic rate may explain the failure of Etomoxir to decrease citrate levels. The PDH complex may, therefore, still be indirectly activated by Etomoxir, as shown by Rosen and Reinauer (1984) who demonstrated an increase in glucose oxidation rates and PDH activity in diabetic rat hearts after perfusion with a related phenylalkyloxirane carboxylic acid, POCA.

The reduced sensitivity of diabetic rat hearts to the effects of Etomoxir on glucose oxidation may be due in part to the increased PDH kinase activity in diabetes, which has been shown by Kerbey et al. (1984). Increased PDH kinase activity in diabetes results in a reduced %PDHa in diabetic rat heart relative to starved or normal rat heart, under various physiologic conditions which increase %PDHa (Randle et al., 1984). CPT I inhibition using tetradecylglycidic acid can normalize %PDHa in starved rat hearts in the presence of

glucose alone, whereas addition of insulin is necessary to reactivate PDH in diabetic rat hearts. While this agent can normalize %PDHa in normal hearts perfused with 0.5 mM palmitate, CPT I inhibition has little effect on %PDHa in palmitate-perfused diabetic rat hearts (Caterson et al., 1982). Thus, either the inhibitory effect of these agents at CPT I may be decreased due to increased competition from stored fatty acids, persisting increased PDH kinase activity, or altered sensitivity of CPT I to inhibitors, in diabetic rat heart.

The effects of Etomoxir on glucose oxidation occurred in the absence of expected decreases in long chain acylcarnitine. Etomoxir is suggested to be a selective CPT I inhibitor, as are other compounds in the same class known as oxirane carboxylic acid derivatives (Wolf et al., 1982). Etomoxir or POCA, acting in the CoA ester form, decreases oxidation rates of palmitoyl CoA but not palmitoylcarnitine when added to isolated liver mitochondria. In isolated liver and muscle mitochondria from POCA-treated animals, oxidation rates of palmitoyl CoA, but not of palmitoylcarnitine, are also reduced (Turnbull et al., 1984). The CoA ester of oxirane carboxylic acid derivatives such as tetradecylglycidate, POCA, and Etomoxir, binds to the CPT I monomer, but not CPT II, in an irreversible manner, thereby inhibiting CPT I, but not CPT II (Declerq et al., 1987). Binding is suggested to occur at the palmitoyl CoA binding site and at another locus which depends on the configuration of the chemical group on carbon 2 (Declerq et al., 1987). The CoA ester is said not to affect citric acid cycle activity or oxidative phosphorylation directly (Turnbull et al., 1984; Eistetter and Wolf, 1986). Recent evidence suggests that

starvation or diabetes can decrease the sensitivity of CPT I to inhibition by the physiologic inhibitor malonyl CoA and to synthetic inhibitors such as 4-hydroxyphenylglyoxylate and POCA in liver mitochondria (Saggerson, 1986; Cook and Gamble, 1987; Grantham and Zammit, 1987, 1988; Stephens and Harris, 1987). One recent report (Fiol et al., 1988) indicates that decreased sensitivity to CPT I inhibition also occurs in heart mitochondria isolated from starved animals. This suggests that the potency of CPT I inhibition by Etomoxir might be reduced in diabetic rat hearts. In the present study, elevated levels of long chain acyl CoA in glucose-perfused diabetic hearts were substantially reduced by Etomoxir. This may be due to reduced mitochondrial fatty acyl CoA levels as a result of transport of fatty acids into the mitochondrion. However, interpretation of these data is complicated by the fact that variations in the levels of CoA in particular cell compartments can be masked by measuring changes in total cellular concentrations.

It was suggested that elevated long chain acyl CoA levels in diabetic hearts perfused with high fatty acid levels could decrease myocardial ATP levels by inhibiting the adenine nucleotide translocase (Paulson and Shug, 1982; Pieper et al., 1984). Pieper and coworkers observed that creatine (CP) phosphate levels remained unchanges in these hearts. In the present study, ATP levels were not reduced in diabetic hearts perfused for one hour in the presence of 1.2 mM palmitate, but CP levels were substantially reduced. Rosen et al. (1986) also observed that disturbed high energy phosphate metabolism was mainly as a result of decreased CP concentrations. It remains unresolved what effect diabetes has on metabolism of high

energy phosphates.

Besides reduced activity of the PDH complex, other factors may play an important role in altering glucose oxidation rates in diabetes and during fatty acid oxidation. Citrate has traditionally been suggested to mediate the effects of fatty acids and diabetes on glycolysis, by inhibiting PFK 1 (Randle et al., 1963; Neely et al., 1970). Fructose-2,6-bisphosphate (Fru-2,6-P2) has been demonstrated as an important regulator of glycolysis in many cell types, including heart and liver (see Hue and Rider, 1987 for review). Fru-2,6-P2 is produced by the PFK 2 reaction and acts as a very potent stimulator of PFK 1, reducing the inhibitory effects of citrate and ATP. Recently the relative roles of various effectors of PFK 1 and glycolysis have been reassessed in relation to the contribution of Fru-2,6-P2. Epinephrine, insulin, and cardiac work were identified as factors which can regulate PFK 1 activity and glycolytic rate, in part by affecting concentrations Fru-2,6-P2 or its stimulatory effect on PFK 1 (Lawson and Uyeda, 1987; Narabayashi et al., 1985). These investigators suggest that variations in glycolytic rate and PFK 1 activity in response to insulin and cardiac work are related to substrate (fructose-6-phosphate) and Fru-2,6-P2 concentrations, and are unrelated to increased citrate levels or alterations in other effectors of PFK 1 such as phosphorylation potential (Lawson and Uyeda, 1987). They suggest that at citrate levels which have been observed during fatty acid oxidation, which can be produced by addition of insulin to glucose-perfused hearts, glycolytic rate and PFK 1 activity would be unaffected. Unpublished work from the laboratory of Louis Hue shows that Fru-2,6-P2 concentration and

glycolytic rate decrease in hearts perfused with ketone bodies, which they suggested was due to the inhibitory effect of citrate on PFK 2 (Hue and Rider, 1987). Thus, the relative roles of citrate and Fru-2,6-P2 in acute regulation of glycolysis by fatty acids are unclear. Fru-2,6-P2 may be involved in long-term regulation of glycolysis under conditions such as starvation and diabetes. Levels of Fru-2,6-P2 are reduced in diabetic rat heart slices (Sochor et al., 1984). The recent work of French et al. (1988) demonstrates that, in contrast to overnight starvation (Rider and Hue, 1984), 48 hour starvation results in a decrease in Fru-2,6-P2 levels which cannot be immediately restored by glucose refeeding and insulin. This suggests that in diabetic rat heart, similar to reduced %PDHa, decreased Fru-2,6-P2 levels would not be increased by short-term treatments to increase glucose uptake or inhibit fatty acid oxidation. In general, the role of fatty acids in regulation of PFK 1 in normal and diabetic rat hearts, particulary by citrate and Fru-2,6-P2 needs further investigation.

CONCLUSIONS

The following summary and conclusions can be drawn from the results of this thesis:

1. Treatment of diabetic rats with DCA significantly increases myocardial glucose oxidation rates in isolated working hearts from diabetic rats perfused with 1.2 mM palmitate. Glucose oxidation rates were increased up to 50-fold in palmitate-perfused, untreated diabetic rat hearts by addition of millimolar concentrations of DCA to the perfusate.

2. In these same hearts, DCA treatment overcame the depressed Ca^{2+}-activated myofibril ATPase activity that was seen in untreated diabetic rats. In the absence of an increase in serum thyroxine levels in DCA-treated diabetics, these data suggest that a metabolic signal related to glucose utilization regulates ATPase activity of cardiac myofibrils.

3. Cardiac function, which is depressed in the diabetic, was not concommitantly increased by DCA treatment. DCA must not have affected variables other than myofibril ATPase, which also regulate cardiac contractility. Reduced heart size and myofibril protein yield as a result of reduced food intake or deleterious effects of the high dose of DCA, may also account for reduced cardiac function in these rats.

4. In isolated working hearts perfused with glucose as the sole substrate, glucose oxidation rates were reduced in 6 week diabetics versus age-matched controls. However, levels of citrate, which is an intermediate of fatty acid oxidation known to inhibit glucose oxidation, were not increased in diabetic rat hearts.

5. The presence of 1.2 mM palmitate resulted in a profound inhibition of glucose oxidation. Inhibition of glucose oxidation upon addition of fatty acids to the perfusate appears to be due to increased fatty acid oxidation compared to glucose-perfused hearts, as suggested by elevated levels of intermediates of fatty acid metabolism. Glucose oxdation was almost completely suppressed in diabetic rat hearts in the presence of palmitate.

6. Rates of $^{14}CO_2$ production from exogenous ^{14}C-palmitate were no different in diabetic and control rat hearts. In these

palmitate-perfused hearts, levels of long chain acylcarnitine, long chain acyl CoA, and citrate were not higher in diabetics versus controls.

7. Addition of the CPT I inhibitor Etomoxir (10^{-6} M) produced an increase in glucose oxidation rates in both the presence and absence of palmitate. However, oxidation of exogenous palmitate was not inhibited by Etomoxir. Nor were levels of long chain acylcarnitine, the product of the CPT I reaction, or citrate, reduced by Etomoxir. Etomoxir may have exerted its effect by increasing flux of pyruvate into the TCA cycle without a decrease in TCA cycle intermediates such as citrate.

8. Cardiac function was reduced in diabetic rat hearts and was increased by Etomoxir in palmitate-perfused control and diabetic rat hearts. This suggests that cardiac function may be compromised during conditions of low glucose utilization such as in the presence of high fatty acid levels and in diabetes. This may be in part acutely reversible by blocking the suppressive effect of fatty acids on glucose utilization with CPT I inhibitors such as Etomoxir.

These studies demonstrate that fatty acids are involved in acute and chronic regulation of glucose oxidation and function in the heart in diabetes.

RECOMMENDATIONS:

The results of these studies suggest many avenues for further research to clarify the role of metabolic disturbances in the myocardium in impairing cardiac function in diabetes.

In terms of the actual metabolic signal which regulates

expression of contractile proteins and other proteins in the heart in diabetes, several studies could be undertaken to further the understanding of the mechanisms involved, as well as the physiologic significance of these changes. From the point of view of the DCA treatment study, an approximation of the in vitro and in vivo PDH activity and levels of Fru-2,6-P2 in diabetic and DCA-treated rats would be enlightening. The relevance of changes in myosin isozyme predominance or myofibril ATPase activity to contractility must be examined in light of the results of the present study. The effect of diabetes and such therapy to improve cardiac metabolism on isomyosins, ATPase activity of myosin and myofibrils, as well as contractility of isolated papillary muscles and isolated working hearts must be coordinately investigated. In this regard, diabetes-induced alterations in other contractile proteins and ion transporting systems should be examined in terms of gene expression and biochemical activity. Examination of the time course of induction of α-MHC mRNA by insulin or Etomoxir in the intact diabetic animal, in relation to the effects of T3 replacement, would provide some insight as to the mechanism of action of the metabolic signal. Regulation of MHC genes by T3 receptor occupancy in diabetes would also be informative regarding the site of interaction between T3 and metabolic signals. Measurement of expression of transfected α-MHC fusion genes under various metabolic conditions, as well as cellular signalling compounds in cultured heart cells would help to further isolate the metabolic mechanisms of MHC induction. Of course, identification of the actual signalling chemical(s) would be an ultimate goal. In terms of the long term control of metabolic

changes in the myocardium in diabetes, cardiomyocyte culture or chronic treatment with CPT I inhibitors such as Etomoxir could be used to examine the time course of changes in levels of Fru-2,6P2 and of PDH "kinase/activator" factor which inhibits PDH in diabetes.

From the point of view of acute effects of fatty acids on glucose oxidation and function in the heart during diabetes, one should investigate the acute effect of high concentrations of palmitate and reversibility by Etomoxir on function in isolated working hearts from diabetic rats at high workload which could be produced by increasing left atrial filling pressure and aortic pressure and/or electrical pacing. This should be carried out in conjunction with measurement of glycolytic and glucose oxidation rates. Measurement of combined endogenous and exogenous fatty acid oxidation rates would add to the understanding of substrate utilization in diabetes. As discussed, this could be performed by radiolabelling the myocardial triacylglycerol pool with subsequent measurement of $^{14}CO_2$ production from stored fatty acids (Paulson and Crass, 1982). Another technique would be to measure specific activity of purified intermediates (e.g., long chain acylcarnitine) common to pathways of exogenous and endogenous fatty acid oxidation, and estimate the dilution of radiolabelled exogenous fatty acids by endogenous fatty acids (Lopaschuk et al., 1986). Adaptation of the system such that simultaneous measurements of myocardial oxygen consumption could be carried out, would allow for calculation of the contribution of carbohydrate and lipid substrates to oxidative metabolism. It would be useful to determine whether a high enough dose of Etomoxir could be used to completely block the acute effects

of palmitate on glucose oxidation in isolated working hearts from control animals. The effects of this concentration of Etomoxir on metabolism and function could then be compared in diabetic rat hearts. Possible defects in high energy phosphate metabolism remain to be clearly identified. A useful approach to investigate this would be measurement of kinetics of ATP synthesis and utilization in perfused hearts by ^{31}P-NMR (Ugurbil et al., 1986). The relative roles of citrate and Fru-2,6-P2 (and other regulators) in control of PFK 1 and glycolysis in the presence of fatty acids and in diabetes should also be clarified.

Finally, as the mechanisms of insulin resistance in diabetes are elucidated by experimental work, clinical trials could be carried out to investigate the effectiveness of the new class of hypoglycemic agents, the phenylalkyloxirane carboxylic acids, in ameliorating metabolic control (Bliesath et al., 1987). Since metabolic control may be related to cardiac dysfunction in diabetic patients (Fisher et al., 1987), it would be useful to describe the relationship between possible improvements in glucoregulation and cardiac function in human diabetics using these hypoglycemic agents.

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APPENDIX A: DETAILED METHODOLOGY

1. Measurement of Substrate Oxidation Rates and Cardiac Function in the Neely Isolated Working Rat Heart Apparatus:

The isolated working rat heart preparation as used in these experiments was adapted from the original methodology developed by J.R. Neely and colleagues in the 1960's (Neely et al., 1967; see Neely and Rovetto for detailed description along with diagrams). The adapted apparatus has an oxygenation chamber which allows for gassing of albumin-containing buffer such that perfusion with long chain fatty acids bound to albumin may be carried out (Morgan et al., 1980). The rig consists of custom-made water-jacketed glassware comoponents and Teflon adapters which allow a tightly sealed system which is required for collection of gaseous $^{14}CO_2$ during measurement of ^{14}C-substrate oxidation. A Haake L water circulator was used to maintain the system at 37 degrees C. Buffer was recirculated by a peristaltic pump (Cole-Palmer, Model 7545) at a flowrate of approx. 120 ml/min. Buffer is pumped to the oxygenation chamber, where it drains through coating the inside surfaces of the chamber, allowing oxygenation but preventing foaming of albumin-containing buffer. The chamber has a gas inlet for carbogen and an outlet which may be connected to a bubble trap for gaseous

CO_2 collection. Near the bottom of the chamber is an overflow outlet which allows free drainage of buffer back into the buffer reservoir under the heart, thereby setting the height of the hydrostatic left atrial filling pressure. From the bottom of the chamber, buffer flows into the left atrium via a right-angled cannula. From the aortic cannula, buffer is pumped by the heart to a compliance chamber whose volume can be adjusted using a plastic syringe which is connected to it. Buffer then is pumped out of the chamber up to an a small chamber which connects to overflow tubing. This aspect of the apparatus differs from that described by Morgan et al. (1980), who describe a needle apparatus connected to the compliance chamber, which creates aortic afterload. Buffer flowing through the needle then drains back into the buffer reservoir. On our apparatus, the height of the overflow chamber determines the aortic hydrostatic afterload against which the heart must work. After reaching the chamber fixed at this height, buffer drains freely back down into the buffer reservoir under the heart. The left atrial and aortic cannulas pass through a custom-made Teflon adaptor, which seals tightly with the heart chamber. A sidearm off of the aortic outflow line is connected to a pressure transducer (Gould P23) which permits measurement of pressure generated by the heart during each cardiac cycle and therefore heart rate. A branch of this line is connected to the Langendorff reservoir located 15 cm above the heart chamber.

GENERAL PERFUSION PROTOCOL:

Rats were anesthetized by sodium pentobarbital injection. The thoracic cavity was then opened with surgical scissors, and the

heart removed and placed in a beaker of cold Langendorff buffer (Krebs Henseleit with 11 mM glucose; see Perfusion Buffers). The heart was then cannulated via the aorta (Langendorff, 1895) and clamped with bulldog clamps as flow from the Langendorff reservoir was started. Extraneous tissue was dissected away using forceps and surgical scissors. The aortic cannulation was secured with sutures, and the pulmonary artery was then cut to allow drainage of coronary effluent. The left atrial opening was located, cannulated, and similarly secured. After this initial preperfusion period of about 10 minutes, the Langendorff line was then clamped, the buffer reservoir placed under the heart, the left atrial line opened, and the afterload line opened. The heart chamber was then sealed to the Teflon adapter, thereby closing the system. The compliance chamber was then adjusted and the gas outflow line placed in the bubble trap. Tracings were recorded from the physiograph (Grass Model 79D) every 5 minutes, and samples were taken for $^{14}CO_2$ every 10 minutes, starting at 10 minutes after function and CO_2 production had assumed a steady state. At the end of perfusion, the seal was broken, buffer flow was clamped, and the heart was freeze-clamped using Wollenberger clamps cooled in liquid nitrogen.

MEASUREMENT OF $^{14}CO_2$ PRODUCTION FROM ^{14}C-SUBSTRATE:

Buffers containing ^{14}C-labelled substrate and 3% bovine serum albumin were prepared as described later (see Perfusion Buffers). $^{14}CO_2$ was collected at 10 minute intervals by simultaneously sampling from: 1) the methylbenzethonium hydroxide bubble for gaseous $^{14}CO_2$ in the form of carbamate, using an automatic pipette, and 2) the perfusion buffer for ^{14}C-bicarbonate

via a resealable rubber sampling port, using a syringe and needle. Buffer samples were immediately stored under mineral oil until later processing. This consisted of injecting a known volume of buffer into a closed metabolic flask containing sulfuric acid, through a rubber stoppered sidearm. A plastic centrewell suspended in the flask contains methylbenzethonium hydroxide. Samples were shaken on a metabolic shaker for 1 hour to so that $^{14}CO_2$ from bicarbonate could be liberated and trapped in the centrewell. The centrewells were then dropped into liquid scintillation vials, to which scintillation fluid (ACS, Amersham) was added, and counted on a liquid scintillation counter (Beckman LS3801). Automatic quench correction by the H# method (external standard) was used to determine dpm values. Substrate oxidation rates were then calculated on an using a program written on Lotus 123 (IBM); see Example Calculation. Total dpm as CO_2 contained in the 25 ml methylbenzethonium hydroxide trap and in the 100 ml perfusion buffer volume were calculated after correction for sample removal. The amount of substrate oxidized could be calculated by dividing $^{14}CO_2$ dpm by the specific activity of ^{14}C-substrate in the buffer (dpm/mol substrate). Oxidative rates were obtained in nmoles glucose oxidized per gram dry heart weight per minute, or nmoes glucose oxidized per minute per heart rate-peak systolic pressure product, as shown in the example calculation.

EXAMPLE CALCULATION FOR SUBSTRATE OXIDATION RATES IN THE ISOLATED WORKING HEART

DATE: JAN 19/87/88 SUBSTRATE OXID RATE I.D.: SW 142 SUBSTRATES: 14C-GLUCOSE

ML BUFFER REMOV PER SMPL (INCL LKS)	INIT HYOH VOL (ML) (mL)	INITIAL BUFFER VOL (mL)	DPM FOR S.A.	#1	#2	#3	MEAN SPEC. ACT. (DPM/uMOL)	DRY HEART WEIGHT(G)
3	25	100	VOL FOR S.A. (L)	145571	130095	134258	136610.3	
	BKG DPM 31		0.0005	CONCN SUBSTR	R.A. (mM)	11	24038.24	0.2475

SMPL VOL	SMPL TIME	DPM#1	DPM#2	AVG DPM -BKG/ HYOH SAMPLE	CV(%) HYOH SAMPLE	AVG DPM -BKG/ 25 mL H_4OH	DPM#1	DPM#2	AVG DPM -BKG/ BUFFER SAMPLE	CV(%) BUFFER SAMPLE	AVG DPM -BKG/ INITIAL BUFF VOL	TOTAL AV DPM
0.5	10	43	49	15	28.284	750	378	361	338.5	3.551	33850	34600
0.5	20	90	95	61.5	5.749	2982	671	942	775.5	24.710	76239	79221
0.5	30	225	231	197	2.154	9215	1416	1435	1394.5	0.963	134425	143640
0.5	40	516	498	476	2.674	21491	1917	1952	1903.5	1.300	180744	202235
0.5	50	1020	1028	993	0.570	43205	3132	4013	3541.5	17.590	324888	368093
0.5	60	1863	1905	1853	1.603	77605	5311	5377	5313	0.878	475465.5	553070.5

PRELOAD: 15CM H2O
A/LOAD: 100CM H2O

COMMENTS:
10-6M ETOMOXIR
@ 30 MINUTES
6 WK CONTROL

TOTAL AV DPM/ MINUTE	NMOL GLUCOSE/ MINUTE	NMOL GLUCOSE/ G DRY.MIN	HR	PSP	HRX PSPX 10-3	NMOL GLUCOSE/ MIN.(HRX PSPX10-3)
0.0						
4462.1	179.6463	725.84	165	110	18.2	9.8979
6441.9	259.3540	1047.90	175	110	19.3	13.4729
5859.5	235.9063	953.16	185	109	20.2	11.6988
16585.8	667.7525	2697.99	200	106	21.2	31.4978
18497.8	744.7286	3009.00	215	102	21.9	33.9594

DIL'N FACTOR	VOL. OUT	DPM SAMPLED R'TOT	COUNTS REMAIN	BUFFER SAMPLE VOLUME	BUFFER DIL'N FACTOR	BUFFER VOL OUT	BUFFER DPM SAMPLED R'TOTAL	BUFFER COUNTS REMAINING
50	0	30	750	1	100	0	1015.5	33850
48	1	153	2952	1	97	3	3342	75223.5
46	2	547	9062	1	94	6	7525.5	131083
44	3	1499	20944	1	91	9	13236	173210.5
42	4	3485	41706	1	88	12	23860.5	311652
40	5	7191	74120	1	85	15	39799.5	451605

PERFUSION BUFFERS:

Stock Solutions for Krebs Henseleit Buffer

Krebs Henseleit Concentrate (10X):

Cpd.	M.W.	Final Concn. (mM)	gm/4L
NaCl	58.4	118	275.65
KCl	74.56	4.7	14.02
KH_2PO_4	136.09	1.2	6.53
$MgSO_4.7H_2O$	246.49	1.2	11.83
$CaCl_2.2H_2O$	147.02	3	17.64

EDTA Stock: 20 mM EDTA (pH to 7.4 with NaOH)

Krebs Henseleit Buffer (11 mM glucose):

	/4L
KH 10X Stock	400 ml
EDTA Stock	100 ml
H_2O	3500 ml
glucose	7.92 g
$NaHCO_3$	8.4 g

Glucose and $NaHCO_3$ are dissolved in a portion of the water and added to the buffer during gassing with carbogen. For no-substrate buffer, eliminate glucose.

Krebs Henseleit Working Heart Buffers:

To make 500 ml of albumin:palmitate containing buffer:

1. Make 2L of no-substrate Krebs Henseleit buffer.

2. Use approx. 250 ml of this buffer to dissolve 15 gms Fraction V bovine serum albumin (BSA; Sigma)(to make 3% BSA solution). Stir on low heat.

3. Dissolve palmitic acid (Kodak) and Na2CO3 to make sodium palmitate:

0.4 mM palmitate: 0.0513 g palmitate + 0.025 g Na2CO3

1.2 mM palmitate: 0.1539 g palmitate + 0.075 g Na2CO3

Add palmitate and sodium carbonate to approx. 15 ml distilled H2O and 8 ml 95% ethanol. Warm solution with stirring until ethanol evaporated and palmitate dissolved. If using ^{14}C-palmitate, seal the solution in a flask connected to another flask on ice, to condense any radioactive vapour.

4. Add sodium palmitate solution to albumin with stirring. Observe clear solution.

5. Dialize overnight at 4 degrees C against remaining Krebs Henseleit no-substrate buffer.

6. Make up working heart buffer on day of perfusion. Make up a large volume of Krebs Henseleit Perfusion Buffer (11 mM glucose). Measure volume BSA:palmitate containing Krebs Henseleit no-substrate buffer dializate. Filter this through a Watman filter under vacuum. Calculate remaining volume of 11 mM glucose Krebs Henseleit buffer to make up to 500 ml working heart buffer. Calculate the remaining amount of glucose needed to make this solution 11 mM (i.e., based on

volume of BSA:palmitate no-substrate buffer). Dissolve this in the Krebs Henseleit glucose buffer and add to BSA:palmitate no-substrate buffer through the filter. If ^{14}C-glucose buffer needed, add solid ^{14}C-glucose along with cold glucose and dissolve. Filter as before.

2. Myofibril ATPase Activity of Rat Ventricle:

MYOFIBRIL ISOLATION (Perry and Corsi, 1958; Belcastro and Sopper, 1984):

Solutions:

Buffer 1: 39 mM Na-borate (pH 7.1); 25 mM KCl; 5 mM EDTA

Buffer 2: 39 mM Na-borate (pH 7.1); 50 mM Tris

Wash Solution: 50 mM Tris (pH 7.4); 5 mM MgCl2; 100 mM KCl; 0.5% Triton X-100

Suspension Medium: 50 mM Tris (pH 7.4); 150 mM KCl

Procedure:

1. Homogenize approx. 100-300 mg powdered ventricular tissue in cold Buffer 1 using a Polytron PT-10 for 2 bursts of 10 seconds at a setting of 7.
2. Centrifuge in a refrigerated centrifuge at 1000Xg.
3. Aspirate the supernatant and discard. Resuspend the pellet in 20 vols Buffer 1 and recentrifuge.
4. Aspirate the supernatant and resuspend the pellet in 20 vols Buffer 2. Centrifuge at 1000Xg as above.

5. Repeat step 4.

6. Repeat steps 4 and 5 except with Wash solution.

7. Resuspend in 20 vols Suspension Medium.

8. Take a small aliquot for protein determination (Lowry et al., 1951).

PROTEIN ASSAY (Lowry et al., 1951):

Stock Solutions:

A1: 2% Na_2CO_3; 0.1 N NaOH

A2: 10% Sodium Dodecyl Sulphate

B1: 1% $CuSO_4$

B2 2% Na Tartrate

C: 50 ml A1, 5 ml A2, 0.5 ml B1, 0.5 ml B2

D: 2 N Folin Phenol Reagent

BSA standard: 1 mg/ml

Procedure:

Standard Curve:

ml BSA Std.	ml H_2O	[BSA](ug/ml)	sample dilution factor
1. 0	0.5		
2. 0.05	0.45	20	X50
3. 0.1	0.4	40	X25
4. 0.2	0.3	80	X12.5
5. 0.25	0.25	100	X10

Make up standards and samples to 0.5 ml with H_2O. Take 2X0.1 ml

aliquots from each standard and sample tube and make up volume to 0.5 with H2O. Add 3 ml Solution C. Let stand 10 minutes. Add 0.3 ml Solution D. Let stand 30 minutes. Read at 750 nm on Pye Unicam PU8800 Spectrophotometer.

MYOFIBRIL ATPase ASSAY (Goodno et al., 1978):

Solutions:

1. Incubation Medium: 4 mM Tris (pH 7.0); 50 mM KCl, 4 mM MgCl2

2. CaCl2:EGTA Solutions in 1 mM imidazole to produce the following free pCa values in the 1 ml reaction volume at pH 7.0 in the presence of 5 mM MgATP, with addition of 0.05 ml Ca-EGTA solution to the assay*: 8, 7, 6.6, 6.3, 6, 5.6, 5.3, and 5 (based on equations of Katz et al., 1970).

pCa	EGTA (uM)	g/250 ml	total calcium (uM)	g/250 ml
5	100	0.1902	166	0.120
5.3			141	0.1036
5.6			118	0.0867
6.0			88	0.0647
6.3			71	0.0522
6.6			54	0.0397
7.0			32	0.0235
8.0			4.3	0.0032

*i.e., in a 1 ml assay volume, with addition of 0.05 ml of solution (i.e., a 20-fold dilution):pCa 4-10 uM-0.2mM free calcium stock solution.

3. 20% Trichloroacetic Acid

4. 50 mM MgATP

5. Suspension Medium (see Myofibril Isolation)

Procedure:

Make up a 1 ml* reaction volume with the following:

0.5 ml incubation medium

0.05 ml Ca-EGTA buffer (5 mM EGTA solution for basal ATPase)

0.25 ml freshly isolated myofibril protein solution (1 mg/ml)

0.1 ml suspension medium

(N.B. final [KCl]=77.5 mM)

*0.9 ml + later addition of 0.1 ml ATP solution

Preincubate at 30 degrees C for 5 minutes.

Assay:

1. Add 1 ml cold TCA solution to X tube.

2. Add 0.1 ml MgATP solution with vortexing at 30 second intervals to each tube.

3. Incubate exactly 5 minutes at 30 degrees C.

4. Stop the reaction by addition to each tube of 1 ml cold TCA solution with vortexing at 30 second intervals.

5. Centrifuge the tubes at 1000Xg and use supernatant for phosphate assay.

PHOSPHATE ASSAY (Taussky and Shorr, 1953):

Solutions:

1. 15 N H_2SO_4

2. Ammonium Molybdate, Ferrous Sulphate Solution: add 0.5 g ammonium molybdate to 5 ml 10 N H_2SO_4. Dissove and make up volume to 8 ml. Add 2.5 g ferrous sulphate and make up volume to 50 ml (fresh daily).

3. Phosphate Standard (1 mM)

Procedure:

1. To blank and standard tubes, add 1.5 ml 12% TCA.

2. To sample tubes (A and X), add 1 ml TCA.

3. Remove 0.05 ml from standard tubes and replace with 0.05 ml phosphate standard (1 mM).

4. To sample tubes, add 0.5 ml supernatant from centrifugation.

5. Add 1 ml of ammonium molybdate-ferrous sulphate solution to all tubes, vortex, and let stand at least 10 minutes.

6. Read OD at 700 nm against water.

Calculation of Myofibril ATPase Activity:

4 X 0.05 umol Pi X [((sample OD)-(xtube OD))/(std. OD)]/0.25 mg prot./5 minutes=.....umoles Pi/(mg prot.min)

3. Tissue Workup and Metabolite Assays:

TISSUE WORKUP*:

1. Weigh vial (4.289 g) containing frozen tissue.
2. Add approx. 400 mg frozen ventricular tissue to cold mortar containing 1.5 ml 6% PCA.
3. Homogenize tissue with pestle immediately.
4. Reweigh vial (3.712 g) to determine the total weight of tissue transferred (0.577 g).
5. Transfer solution to preweighed tube C; save mortar.
6. Centrifuge at 10000 rpm for 10 minutes.
7. Pour supernatant into tube A (for free CoA, citrate).
8. Rinse mortar and pestle with 4 ml (2X2 ml) 1.2 % PCA and pour onto pellet.
9. Break up pellet with glass rod and transfer to a glass homogenizer.
10. Homogenize, pour into tube C (for long chain acylcarnitine (LCAC) and long chain acyl CoA (LCACoA); rinse homogenizer with 2X2 ml 1.2% PCA.
11. Spin at 10000 rpm for 10 minutes.
12. Resuspend pellet in 6 ml 2% PCA. Stir with glass rod.
13. Spin again at 10000 rpm for 10 minutes.

*N.B. weights in parentheses are those used for example calculation SW 174.

WORKUP FOR SUPERNATANT (Tube A):

Weigh Tube A (9.514 g)

Add 0.2 ml 1 M MOPS

+95 ul 0.32 M DTT

Neutralize with KOH

Weigh tube (9.992 g)

Centrifuge at 10000 rpm

for 10 minutes----------->Dry Precipitate

Supernatant contains Citrate, Weigh tube A (8.319 g)

ATP, CP

WORKUP FOR PELLET (Tube C):

Pellet is in preweighed tube C (8.304 g)

Add 1 ml 30 mM DTT in KH_2PO_4 buffer

Resuspend pellet

Weigh tube C (9.839 g)

Take 0.5 ml suspension;

put in preweighed tube D (8.330 g)(for LCAC)

Weigh tube D (8.818 g);

Remaining suspension in tube C------->For LCACoA isolation:

Weigh tube C

(9.314 g)

Add 0.5 ml H_2O to tube D

to make 15 mM DTT

pH to 13 with 7.5 N KOH (1 drop)

Hydrolyze at 70 degr. C for 1 hour

Lower pH to 1 with 50 ul 70% PCA

Weigh tube D (9.440 g)

(continued next page)

Add 0.5 ml 30 mM DTT

Neutralize to pH 7 with KOH

Weigh tube C (9.991 g)

Boil 10 min

Spin at 10000 rpm 10 min

Dry pellet;

weigh tube C (8.385 g)

Supernatant contains LCACoA

(Processing for LCAC in Tube D, continued from previous page):

Spin at 10000 rpm 10 min----------->Dry pellet

Supernate into and weigh D (8.402 g)

preweighed tube E(8.512g)

Weigh tube E (9.309 g)

Add 100 ul 1 M MOPS

Neutralize with KOH

Weigh tube E(9.508 g)

Spin 10000 rpm 10 min----------->Dry Pellet

Supernatant contains LCAC and weigh E (8.551 g)

Calculations:

Citrate*:

[(corrected neutral vol.)/(supernatant) X (1.5 ml+tissue H20)/
(g dry wt.)-ml/g dry weight] X umoles/mol (from assay)-
umoles/g dry weight.

e.g.,SW 174

((9.992-8.319)/(9.514-8.319))X((1.5+(0.577-0.083))/0.08309)-
30.26 ml/g dry wt.X0.104 umol/ml (from citrate assay)-
3.14 umol/g dry wt.

Long Chain Acyl CoA:

(corrected neutral vol.)/(supernatant) X (pellet before/pellet after)
X(1/g dry wt.)-ml/g dry wt.

e.g., SW 174

((9.991-8.385)/((9.314-8.385)+0.5))X((9.839-8.304)/(9.314-8.304))
X(1/0.08309)-20.6 ml/g dry wt.X6.53 nmol/ml-143.6 nmol/ g dry wt.

Long Chain Acyl Carnitine:

(pH1-dry)/(supernatant)X(neutral-dry)X(pellet before/pellet transf.)
X(1/g dry)-ml/g dry wt.

e.g., SW 174

((9.440-8.402)/(9.309-8.512))X((9.508-8.551)/1)
X((9.839-8.304)/(8.818-8.330))X(1/0.0839)-47.2 ml/g dry wt.
X9.50 nmol/ml-448.4 nmol/g dry wt.

*see ASSAY METHODOLOGY (next page).

N.B. use same calculations as for citrate for ATP and CP.

METABOLITE ASSAY METHODOLOGY:

Long Chain Acylcarnitine (McGarry and Foster, 1976):

Principle:

L-carnitine + 3H-acetyl CoA-------->3H-acetyl-L-carnitine + CoASH

(CAT)

CAT-carnitine acetyltransferase.

Reagents:

HEPES buffer 120 mM (pH 7.3)

Assay Buffer: Acetyl CoA 0.45 mg/50 ml

Carnitine acetyltransferase 62.5 ul (U?)/50 m

Na Tetrathionate (reducing agent) 18.3 mg/50 ml

^{3}H-acetyl CoA 1 uCi/50 ml

Procedure:

1. Add 1 ml buffer to 200 ul sample or standard in Eppendorf tube (range 0-4 nmol; linear up to approx. 5 nmol).
2. Shake once.
3. Incubate for 30 minutes at room temp.
4. Add 300 ul of Dowex to tube.
5. Shake, incubate 10 minutes, Repeat. Shake.
6. Spin at 14000 rpm in Eppendorf microcentrifuge for 10 min.
7. Add 750 ul supernatant (contains 3H-acetyl carnitine) to 20 ml scintillation vials.
8. Add 10 ml scintillation fluid and count.

Dowex Columns:

Dowex 1 (200-400 mesh: chloride anion exchanger).

Activate with 1 N HCl. Pass through filter. Wash with H2O until

neutral pH 5-7 (use Litmus paper). Filter to dryness. Store in beaker refrigerated.

Day of use: Mix Dowex with H2O 1:2. 10 g/20 ml. To each Eppendorf tube add 300 ul of slurry.

Long Chain Acyl CoA (Tubbs and Garland, 1969):

Principle:

CoASH + alpha-ketoglutarate + NAD-------->succinyl CoA + CO2 + NADH

(alphaKGDH)

Reagents:

Tris HCl buffer (pH 6.8)

0.1 mM NADH Standard Solution in Tris buffer (keep frozen)

alpha-ketoglutarate dehydrogenase

NaCN stock, 0.1 M

MOPS stock, 1 M pH 6.8

CoA Buffer: per 50 ml

NAD 12.5 mg

alpha-ketoglutarate 5 mg

NaCH Stock 1 ml

H2O 46.5 ml pH to 6.85

NADH Standardization:

Determine OD of 0.1 mM NADH solution at 340 nm. The exact NADH concentraiton can then be determined useing the extinction coefficient of NADH (6.22). 10 ul will be added to fluorimetric assay solution to calibrate the amount of NADH produced in the assay under the sensitivity conditions used.

e.g., if OD=0.59, then 0.59/0.622=0.949 nmoles/ml

-if 0.010 ml NADH reads 16.5 units on the fluorimeter, then a constant can be derived, k=0.949/16.5=0.0575

-if a 0.1 ml sample is added to the assay and the fluorescence change is 11 units, the concentration of long chain acyl CoA can be calculated as:

11X0.0575X10-6.325 nmoles/ml.

Procedure:

Run assay on Perkin Elmer Fluorescence Spectrophotometer as at 340 nm excitation, and 640 nm emmision.

Set scale to get about 15-30 units per 10 ul of 0.1 mM NADH solution in assay volume.

Assay contents:

2.5 ml CoA Buffer

100 ul sample

Zero the machine. Take integrated reading.

Add 10 ul alpha-KGDH

Follow reaction on chart recorder until completion.

Take integrated reading. Add 10 ul enzyme. Take reading and calculate change in fluorescence due to reaction (subtract enzyme fluorescence).

Add 10 ul NADH solution and observe difference due to NADH.

Adenosine Triphosphate, Creatine Phosphate (Lamprecht and Stein, 1974):

Use Neutralized PCA Extract (i.e., tube A from Tissue Workup).

Principle:

ADP + CP------------------>ATP + Creatine

(CPK)

Glucose + ATP-------------->G6P + ADP

(HK)

G6P + NADP----------------->6 phosphogluconate + NADPH

(G6PDH)

Reagents:

Reaction Buffer: 100 ml 50 mM HEPES, 10 mM MgCl2, 5 mM EDTA pH 7.4

NADP 25 mg

Glucose 45 mg

G6PDH (300 U/ml) 200 ul

ADP 3.8 mg

Hexokinase 3 U/assay

Creatine Phosphokinase 10 U/assay

Procedure:

Add 1 ml buffer and 50-100 ul sample. Read E1 at 340 nm.

Add 10 ul HK. Let reaction run to completion. Read E2.

Add 10 ul CPK. Let reaction run to completion. Read E3.

ATP=E2-E1.

CP=E3-E2.

Calculation: (deltaE X assay vol)/(6.22Xsample vol)=umoles/ml

Citrate (Dagley, 1974):

Use neutralized PCA extract from tube A.

Principle:

Citrate----------->OAA + Acetate

(CL)

OAA----------->Pyruvate + CO2

Pyruvate + OAA + NADH--------->Lactate + Malate + NAD

(LDH,MDH)

Reagents:

0.1 M Tiethanolamine (TRA), 0.2 mM ZnCl2 Buffer pH 7.6

NADH 10 mM in TRA buffer

MDH (2200 U/ml) 230 ul

LDH (1100 U/ml) 110 ul: bring up to 1 ml TRA buffer

Citrate Lyase 50 U/500 ul

Ammonium Sulphate 3.2 M

Procedure:

Reaction Mixture:

50 ul LDH/MDH

60 ul NADH

2.8 ml TRA buffer

100 ul sample

Mix Read E1 at 340 nm.

Add 20 ul CL. Let reaction run to completion. Read E2.

E1-E2=OD due to citrate.

Calculation: (delta E X reaction vol)/(6.22Xsample vol)=umol/ml

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